(54) Title: USE OF CROSSLINKED CRYSTALS AS A NOVEL FORM OF ENZYME IMMOBILIZATION

(57) Abstract

A method of immobilizing an enzyme by forming crystals of the enzyme and, generally, also cross-linking the resulting crystals through the use of a bifunctional reagent; crosslinked, immobilized enzyme crystals (CLECs) made by this method; lyophilization of CLECs made by this method; lyophilized crosslinked immobilized CLECs and a method of making a desired product by means of a reaction catalyzed by a CLEC or set of CLECs.

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USE OF CROSSLINKED CRYSTALS AS A NOVEL FORM OF ENZYME IMMOBILIZATION

Related Application

This application is a Continuation-in-Part of
5   U.S.S.N. 07/562,280 filed August 3, 1990 and entitled
"Use of Crosslinked Crystals as a Novel Form of Enzym
Immobilization". The teachings of U.S.S.N. 07/562,280
are incorporated herein by reference.

Background of the Invention

10   Enzymes are used as industrial catalysts for the
large and laboratory scale economical production of
fine and specialty chemicals (Jones, J.B., Tetrahedr n
42: 3351-3403 (1986)), for the production of foodstuffs
(Zaks et. al., Trends in Biotechnology 6: 272-275
15   (1988)), and as tools for the synthesis of organic
compounds (Wong, C.-H., Science 244: 1145-1152 (1989);
CHEMTRACTS-Org. Chem. 3: 91-111 (1990); Klibanov,
A.M., Acc. Chem. Res. 23: 114-120 (1990)).
        Enzyme-based manufacturing can significantly
20   reduce the environmental pollution burden implicit in
the large scale manufacturing of otherwise unusable
chemical intermediates, as shown in the large scale
production of acrylamide using the enzyme, nitrile
hydratase (Nagasawa, T. and Yamada, H., Trends in
25   Biotechnology 7: 153-158 (1989)).
        Enzymes are also used in biosensor applications to
detect various substances of clinical, industrial and
other interest (Hall, E., "Biosensors", Open University
Press (1990)). In the clinical area, enzymes may be
30   used in extracorporeal therapy, such as hemodialysis
and hemofiltration, where the enzymes selectively
remove waste and toxic materials from blood (Klein, M.

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and Langer, R., Tr nds in Biotechn logy 4: 179-135
(1986)). Enzymes are used in these areas because they
function efficiently as catalysts for a broad range of
reaction types, at modest temperatures, and with
5 substrate specificity and stereoselectivity.
Nonetheless, there are disadvantages associated with
the use of soluble enzyme catalysts which have limited
their use in industrial and laboratory chemical
processes (Akiyama et. al., CHEMTECH 627-634 (1988)).
10   Enzymes are expensive and relatively unstable
compared to most industrial and laboratory catalysts,
even when they are used in aqueous media where enzymes
normally function. Many of the more economically
interesting chemical reactions carried out in common
15 practice are incompatible with aqueous media, where,
for example, substrates and products are often
insoluble or unstable, and where hydrolysis can compete
significantly. In addition, the recovery of soluble
enzyme catalyst from product and unreacted substrate in
20 the feedstock often requires the application of compli-
cated and expensive separation technology. Finally,
enzymes are difficult to store in a manner that retains
their activity and functional integrity, for commer-
cially reasonable periods of time (months to years)
25 without having to resort to refrigeration (4°C to -80°C
to liquid N_2 temperatures), or to maintenance in
aqueous solvents of suitable ionic strength, pH, etc.
     Enzyme immobilization methods have, in many
instances, circumvented these disadvantages.
30 Immobilization can improve the stability of enzyme

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catalysts and protect their functional integrity in the harsh solvent environments and extreme temperatures characteristic of industrial and laboratory chemical processes (Hartmeier, W., Trends in Biotechnology 3:
5 149-153 (1985)). Continuous flow processes may be operated with immobilized enzyme particles in columns, for example, where the soluble feedstock passes over the particles and is gradually converted into product.

As used herein, the term enzyme immobilization
10 refers to the insolubilization of enzyme catalyst by attachment to, encapsulation of, or by aggregation int macroscopic (10^{-1} mm) particles.

A number of useful reviews of enzyme immob- ilization methods have appeared in the literature
15 (Maugh, T.H., Science 223: 474-476 (1984); Tramper, J., Trends in Biotechnology 3: 45-50 (1985)). Maugh describes five general approaches to the immobilizati n of enzymes. These include: adsorption on solid supports (such as ion-exchange resins); covalent
20 attachments to supports (such as ion-exchange resins, porous ceramics or glass beads); entrapment in poly- meric gels; encapsulation; and the precipitation of soluble proteins by cross-linking them with bifunc- tional reagents in a random and undefined manner. In
25 addition, one can immobilize whole cells (usually dead and made permeable) which have expressed the desired enzyme activity at high levels (e.g., Nagasawa, T. and Yamada, H., Trends in Biotechnology 7: 153-158 (1989)).

Each of these immobilization procedures has its
30 own advantages and limitations and none can be

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considered optimal or dominating. In most of them, the
enzyme catalyst ultimately represents only a small
fraction of the total volume of material present in the
chemical reactor. As such, the bulk of the immobilized
5   medium is made up of inert, but often costly carrier
material. In all of them, the immobilizing
interactions of the enzyme catalyst molecules with each
other and/or with the carrier material tend to be
random and undefined. As a result, although these
10 interactions confer some enhanced stability to the
enzyme catalyst molecules, their relative
non-specificity and irregularity makes that
stabilization sub-optimal and irregular. In most
cases, access to the active site of the enzyme catalyst
15 remains ill-defined. In addition, the immobilizati n
methods described above fail to deal with problems
associated with storage and refrigeration. Nor can
conventionally immobilized enzymes generally be
manipulated, as in being exchanged into one or another
20 solvent of choice, without risk to the structural and
functional integrity of the enzyme. In practical
terms, except for the attached tether to the carrier
particle, conventionally immobilized enzymes bear cl se
resemblance to soluble enzymes, and share with them a
25 susceptibility to denaturation and loss of function in
harsh environments.   In general, immobilization
methods lead to a reduction of observed
enzyme-catalyzed reaction rates relative to those
obtained in solution. This is mostly a consequence of
30 the limits of inward diffusion of substrate and outward

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diffusion of product within the immobilized enzyme particle (Quiocho, F.A., and Richards, F.M., Biochemistry 5: 4062-4076 (1967)). The necessary presence of inert carrier in the immobilized enzyme
5 particles increases the mean free path between the solvent exterior of the immobilized enzyme particle and the active site of the enzyme catalyst and thus exacerbates these diffusion problems. When dealing with immobilized cells, the diffusion problem is
10 particularly severe, even if cell walls and membranes are made permeable to substrate and product in some way. One would further be concerned with the multitude of contaminating enzymatic activities, metabolites, and toxins contained in cells, and with the stability of
15 cells in harsh solvents or extreme temperature operating environments. An improved immobilization technique which avoids the limitations of the presently available methods would be helpful in promoting the use of enzymes as industrial catalysts, particularly if it
20 were shown to be useful on a large scale (Daniels, M.J., Methods in Enzymology 136: 371-379 (1987)).

Summary of the Invention

The present invention relates to a method of immobilizing an enzyme by forming crystals of the
25 enzyme and, generally, also cross linking the resulting crystals through use of a bifunctional reagent; cross-linked immobilized enzyme crystals (referred to as CLECs or CLIECs) made by this method; the lyophilization of CLECs as a means of improving the

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storage, handling, and manipulation properties of
immobilized enzymes and a method of making a desired
product by means of a reaction catalyzed by a CLEC or a
set of CLECs.

5   In the method of the present invention, small
(10^{-1}mm) protein crystals are grown from aqueous
solutions, or aqueous solutions containing organic
solvents, in which the enzyme catalyst is structurally
and functionally stable.   In a preferred embodiment,
10   crystals are then cross-linked with a bifunctional
reagent, such as glutaraldehyde. This cross-linking
results in the stabilization of the crystal lattice
contacts between the individual enzyme catalyst
molecules constituting the crystal. As a result of
15   this added stabilization, the cross-linked immobilized
enzyme crystals can function at elevated temperatures,
extremes of pH and in harsh aqueous, organic, or
near-anhydrous media, including mixtures of these.
That is, a CLEC of the present invention can function
20   in environments incompatible with the functional
integrity of the corresponding uncrystallized,
uncrosslinked, native enzyme or conventionally
immobilized enzyme catalysts.

In addition, CLECs made by this method can be
25   subjected to lyophilization, producing a lyophilized
CLEC which can be stored in this lyophilized form at
non-refrigerated (room) temperatures for extended
periods of time, and which can be easily reconstituted
in aqueous, organic, or mixed aqueous-organic solvents
30   of choice, without the formation of amorphous

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suspensions and with minimal risk of denaturation.

The present invention also relates to CLECs produced by the present method and to their use in laboratory and large scale industrial production of

5 selected materials, such as chiral organic molecules, peptides, carbohydrates, lipids, or other chemical species. Presently, these are typically prepared by conventional chemical methods, which may require harsh conditions (e.g. aqueous, organic or near-anhydrous

10 solvents, mixed aqueous/organic solvents or elevated temperatures) that are incompatible with the functional integrity of uncrystallized, uncrosslinked, native enzyme catalyst. Other macromolecules with catalytic activity can also be incorporated into the proposed

15 CLEC technology. These might include catalytic antibodies (Lerner, R.A., Benkovic, S.J., and Schultz, P.G., Science 252:659-667 (1991)) and catalytic polynucleotides (Cech, T.R., Cell 64:667-669 (1991); Celander, D.W., and Cech, T.R. Science, 251:401-407

20 (1991)).

The present invention also relates to a method of making a selected product by means of a reaction catalyzed by a CLEC of the present invention.

In an example of the method and practice of the

25 present invention, the enzyme thermolysin, a zinc metalloprotease, was used to synthesize a chiral precursor of the dipeptidyl artificial sweetener, aspartame. The enzyme thermolysin was crystallized from a starting aqueous solution of 45% dimethyl

30 sulfoxide, and 55% 1.4M calcium acetate, 0.05M sodium

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cacodylate, pH 6.5. The resulting crystals were
cross-linked with glutaraldehyde to form a thermolysin
CLEC. The thermolysin CLEC was then transferred fr m
the aqueous crystallization solution in which it was
5   made, into a solution of ethyl acetate containing the
substrates, N-(benzyloxycarbonyl)-L-aspartic acid
(Z-L-Asp) and L-phenylalanine methyl ester (L-Phe-OMe).
The thermolysin CLEC was then used to catalyze a
condensation reaction of the two substrates to
10   synthesize N-(benzyloxycarbonyl)-L-aspartyl-
L-phenylalanine methyl ester (Z-L-Asp-L-Phe-OMe), which
is the dipeptidyl precursor of the artificial sweetener
aspartame. Using any one of many known techniques
(see, e.g. Lindeberg, G., J. Chem Ed. 64: 1062-1064
15   (1987)) the L-aspartic acid in the synthesized
dipeptidyl precursor can be deprotected by the removal
of the benzyloxycarbonyl (Z-) group to produce
aspartame (L-Asp-L-Phe-OMe).

In a second example of the method and practice of
20   the present invention, the enzyme thermolysin was used
to produce thermolysin CLECs. The activity and
stability of thermolysin CLECs were compared to that f
soluble thermolysin under optimum conditions and
conditions of extreme pH and termperature, following
25   incubation in the presence of organic solvents and
following incubation in the presence of exogenous
protease.

The enzyme thermolysin was crystallized from a
solution of 1.2M calcium acetate and 30% dimethyl
30   sulfoxide pH 8.0. The resulting crystals were

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crosslinked with glutaraldehyde at a concentration f
12.5% to form a thermolysin CLEC. The thermolysin CLEC
was then lyophilized by a standard procedure (Cooper,
T.G., _The Tools of Biochemistry_, pp. 379-380 (John
5   Wiley and Sons, NY (1977)) to form a lyophilized enzym
CLEC of thermolysin. This lyophilized CLEC was then
transformed directly into the different aqueous,
organic, and mixed aqueous/organic solvents of choice
without an intervening solvent exchange procedure,
10 without formation of amorphous suspensions, and with
minimal risk of denaturation. These solvents included
acetonitrile, dioxane, acetone, and tetrahydrofuran,
but not to the exclusion of others. Following
incubation, activity was assayed spectrophotometrically
15 by cleavage of the dipeptide substrate FAGLA
(furylacryloyl-glycyl-L-leucine amide).
        In a third example of the method and practice of
the present invention, the enzyme elastase (porcine
pancreatic) was crystallized from an aqueous solution
20 of 5.5 mg/ml protein in 0.1 M sodium acetate at pH 5.0
at room temperature (Sawyer, L. et al., J. Mol. Biol.
118:137-208). The resulting crystals were crosslinked
with glutaraldehyde at a concentration of 5% to form an
elastase CLEC. The elastase CLEC was lyophilized as
25 described in Example 2.
        In a fourth example of the method and practice f
the present invention, and as disclosed here, the
enzyme esterase (porcine liver) was crystallized from
an aqueous solution of 15 mg/ml protein in 0.25 M
30 calcium acetate at pH 5.6 at room temperature. The

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resulting crystals were crosslinked with glutaraldehyde
at a concentration of 12.5% to form an esterase CLEC.
The esterase CLEC was lyophilized as described in
Example 2.

5   In a fifth example of the method and practice f
the present invention, and as disclosed here, the
enzyme lipase (Geotrichum candidum) was crystallized
from an aqueous solution of 20 mg/ml protein in 50 mM
Tris at pH 7 at room temperature. The resulting
10   crystals were crosslinked with glutaraldehyde at a
concentration of 12.5% to form a lipase CLEC. The
lipase CLEC was lyophilized as described in Example 2.

        In a sixth example of the method and practice of
the present invention, the enzyme lysozyme (hen egg
15   white) was crystallized from an aqueous solution of 40
mg/ml protein in 40 mM sodium acetate buffer containing
5% sodium chloride at pH 7.4 at room temperature
(Blake, C.C.F. et al., Nature, 196:1173 (1962)). The
resulting crystals were crosslinked with glutaraldehyde
20   at a concentration of 20% to form a lysozyme CLEC. Th
lysozyme CLEC was lyophilized as described in Example
2.

        In a seventh example of the method and practice of
the present invention, the enzyme asparaginase
25   (Escherichia coli) was crystallized from an aqueous
solution of 25 mg/ml protein in 50 mM sodium acetate
and 33% ethanol at pH 5.0 at 4°C. The crystallizati n
is a modification of the procedure described by Grabner
et al. [U.S. Patent 3,664,926 (1972)]. As disclosed
30   here, the resulting crystals were crosslinked with

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glutaraldehyde at a concentration of 7.5% to form an
asparaginase CLEC. The asparaginase CLEC was
lyophilized as described in Example 2.

Other enzymes which can be immobilized in a
5 similar manner and used to catalyze an appropriate
reaction include luciferase and urease. Other enzymes,
such as those listed in Tables 1-5, can also be
crystallized and crosslinked using the present method,
to produce a desired CLEC which can, in turn, be used
10 to catalyze a reaction which results in production of a
selected product or to catalyze a reaction which is an
intermediate step (i.e. one in a series of reactions)
in the production of a selected product. It is
recognized that although crosslinking helps stabilize
15 the majority of crystals, it is neither necessary nor
desirable in all cases. Some crystalline enzymes
retain functional and structural integrity in harsh
environments even in the absence of crosslinking.
Although in the preferred embodiment, the crystal is
20 crosslinked, crosslinking is not always necessary to
produce an enzyme crystal useful in the present method.

CLECs have several key characteristics that confer
significant advantages over conventional enzyme
immobilization methods presently in use. CLECs
25 dispense with the need for a separate, inert support
structure. Lack of an inert support will improve
substrate and product diffusion properties within CLECs
and provides enzyme concentrations within the crystal
that are close to the theoretical packing limit for
30 molecules of such size. High enzyme concentrations

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can lead to significant operational economies through
the increased effective activity of a given volume f
catalyst, reduction in substrate contact time with
enzyme and overall reduction in plant size and capital

5   costs (Daniels,M.J., Methods in Enzymol. 136: 371-379
(1987)).   The uniformity across crystal volume and
enhanced stability of the constituent enzyme in CLECs
creates novel opportunities for the use of enzyme
catalysis in harsh conditions, such as elevated

10   temperature, and aqueous, organic or near-anhydrous
solvents, as well as mixtures of these. In addition,
the restricted solvent access and regular protein
environment implicit in a crystal lattice should lead
to improved metal ion and co-factor retention for CLECs

15   vs. conventional immobilized enzyme systems.

Brief Description of the Drawings

        Figure 1 is a graphic representation of results f
assessment of enzymatic activity of soluble and
thermolysin CLEC.

20   Figure 2 is a graphic representation of results f
a comparison of pH dependencies of thermolysin CLEC
and soluble thermolysin.

        Figure 3 is a graphic representation of
measurement of the activity of soluble and crystalline

25   thermolysin after incubation at 65°C.

        Figure 4 is a graphic representation of results of
assessment of resistance of soluble and thermolysin
CLEC to exogenous proteolytic degradation.

        Figure 5 is a graphic representation of results f

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the assessment of enzymatic activity for soluble elastase and the corresponding elastase CLEC.

Figure 6 is a graphic representation of the resistance of soluble elastase and the corresponding

5 elastase CLEC to exogenous proteolytic degradation.

Figure 7 is a graphic representation of results of the assessment of enzymatic activity for soluble esterase and the corresponding esterase CLEC.

Figure 8 is a graphic representation of the

10 resistance of soluble esterase and the corresponding esterase CLEC to exogenous proteolytic degradation.

Figure 9 is a graphic representation of results f the assessment of enzymatic activity for soluble lipase and the corresponding lipase CLEC.

15   Figure 10 is a graphic representation of results of the assessment of enzymatic activity for soluble lysozyme and the corresponding lysozyme CLEC.

Figure 11 is a graphic representation of results of the assessment of enzymatic activity for soluble

20 asparaginase and the corresponding asparaginase CLEC.

Detailed Description of the Invention

A simple, general procedure that assures stability and function for a given enzyme or set of enzymes under conditions which are of interest to the synthetic

25 chemist and which are too harsh for use with enzymes using presently available methods, would be very useful. Cross-linked immobilized enzyme crystals (referred to as CLECs or CLIECs) as described here can be used for this purpose. Stabilization of the crystal

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lattice and of the constituent enzyme catalysts in the
crystal by the cross-linking reaction permits the use
of CLECs in environments, including aqueous, organic
or near-anhydrous solvents, mixtures of these solvents,
5   extremes of pH and elevated temperatures, which are
incompatible with enzyme function using presently
available methods. In addition, the stabilization f
the crystal lattice in CLECs makes possible the
lyophilization of CLECs by standard methods.
10   Lyophilized CLECs can be stored for commercially
attractive periods of time (months to years) in the
absence of refrigeration, and facilitate the rapid and
uncomplicated utilization of CLECs in industrial and
laboratory scale processes by the simple addition of
15   solvents of choice, without need for intervening
solvent exchange processes. CLECs are also highly
resistant to digestion by exogenous proteases. The
method of the present invention facilitates the use of
versatile enzyme catalysts in mainstream industrial
20   chemical processes, as well as in the laboratory
synthesis of novel compounds for research.

        Although crosslinking contributes to the stability
of a crystal enzyme, it is neither necessary nor
desirable in all cases. Some crystallized enzymes
25   retain functional and structural integrity in harsh
environments even in the absence of crosslinking. The
preferred embodiment of the present method includes
cross-linking of a crystal enzyme and is described in
detail in the following sections. It is to be
30   understood, however, that crystallized enzymes which

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are not subsequently cross-linked can be used in some embodiments of the present invention.

The regular interactions between the constituent enzyme molecules in the crystal lattice of a CLEC

5 result in well defined pores of limited size leading to the enzyme molecules within the body of a CLEC. As a result, substrates larger than the available pore size will not penetrate the body of the CLEC particle.

As a consequence of the limited pore size, many

10 enzymatic reactions of commercial and academic interest involving substrates larger than the pore size of the CLECs would be beyond the scope of the present invention. This would include most reactions involving large polymers, such as proteins, polynucleotides,

15 polysaccharides, and other organic polymers, where the number of polymeric subunits would be such as to make the polymer larger than the crystal pore size in CLECs. In such instances, however, catalysis can still take place on the CLEC surface.

20 The present invention is a method of immobilizing a selected protein, particularly an enzyme, by crystallizing and crosslinking the protein, resulting in production of a crosslinked immobilized enzyme crystal (CLEC) which can be used to catalyze production

25 of a selected product, such as a peptide, carbohydrate, lipid or chiral organic molecule. The present invention further relates to such CLECs and to a method of making a selected product by means of a CLEC-catalyzed reaction or CLEC-catalyzed step in a

30 series of reactions. In one embodiment of the present

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invention, the dipeptidyl precursor of aspartame has
been produced in a condensation reaction catalyzed by
cross-linked immobilized thermolysin made by the
present method. In another embodiment of this
5 invention, the indicator substrate, FAGLA, has been
cleaved to produce a colorimetric product, whose
presence is indicative of enzyme activity in a
thermolysin CLEC. FAGLA hydrolysis has been used as a
model reaction to indicate the robustness of the
10 thermolysin CLEC in a number of environments that would
be normally incompatible with that enzyme's activity.

In other embodiments of this invention, the
enzymes elastase, esterase, lipase, asparaginase, and
lysozyme have been used to cleave various indicated
15 substances, such as p-nitrophenyl acetate (esterase and
lipase), succinyl- (ala)3-p-nitroanilide (elastase), 4-
methylumbelliferyl N-acetyl-chitrioside (lysozyme) and
NADH (asparaginase).

By the method of this invention, one of ordinary
20 skill in the art can adapt a protocol for making a
desired product by means of a reaction catalyzed by an
immobilized enzyme. The enzyme of interest, when
crystallized from an appropriate solution, can be
cross-linked with glutaraldehyde or other suitable
25 bifunctional reagent in the crystallization solution to
produce a CLEC of that enzyme. Subsequently, the CLEC
of the enzyme of choice can be lyophilized as described
in Example 2.

There are several advantages which the use of a

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CLEC offers over presently-available enzyme-catalyzed methods. For example, the cross-linked crystal matrix in a CLEC provides its own support. Expensive carrier beads, glasses, gels, or films are not required in

5   order to tie down the enzyme catalyst, as they are in presently-available immobilization methods. As a result, the concentration of enzyme in a CLEC is close to the theoretical packing limit that can be achieved for molecules of a given size, greatly exceeding

10   densities achievable even in concentrated solutions. The entire CLEC consists of active enzyme (and not inactive carrier), and thus, the diffusion-related reduction of enzyme reaction rates usually observed with conventionally immobilized enzymes relative to

15   enzymes in solution should be minimized, since the mean free path for substrate and product between active enzyme and free solvent will be greatly shortened f r CLECs (compared to a conventional immobilized enzyme carrier particles). These high protein densities

20   will be particularly useful in biosensor, analytical and other applications requiring large amounts of protein in small volumes. In industrial processes, the superior performance and compactness of CLECs results in significant operating economies, by

25   increasing the effective activity of a given volume of catalyst, thereby allowing reductions in plant size, as well as capital costs (Daniels, M.J., Methods in Enzymol. 136: 371-379 (1987)). CLECs are relatively monodisperse, with a macroscopic size and shape

30   reflecting natural crystal growth characteristics of

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the individual enzyme catalysts. Replacement of
existing carrier-immobilized enzyme media with CLECs
should not be difficult, since both systems are
comparable in size and shape, and both can be similarly
5   recovered from feedstock by any number of simple
methods, including basic economical operations such as
filtration, centrifugation, decantation of solvent, and
others.

In addition, the use of lyophilized CLECs permits
10   routine handling and storage of these materials prior
to use (dry storage at room temperature without
refrigeration, for extended periods of time).
Lyophilized CLECs also allow for routine formulation by
direct addition of solvents and substrates of inter st,
15   without lengthy solvent exchange processes, or the
formation of amorphous suspensions. The lyophilized
CLEC form extends the general utility of the enzymes as
catalysts to a broader spectrum of enzymes and
functional conditions.
20   A second advantage of a CLEC is that cross-linking
of the crystallized enzyme stabilizes and strengthens
the crystal lattice and the constituent enzyme
molecules, both mechanically and chemically. As a
result, a CLEC may be the only means of achieving
25   significant concentrations of active enzyme catalyst in
harsh aqueous, organic, near-anhydrous solvents,   r in
aqueous-organic solvent mixtures. The use of enzym s
as catalysts in organic syntheses has been hampered by
their tendency to denature in the presence of
30   non-aqueous solvents, and particularly, in mixtures f

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aqueous and non-aqueous solvents (Klibanov, A.M.,
Trends in Biochemical Sciences, 14:141-144 (1989)). In
CLECs, the restriction of conformational mobility that
leads to stability is provided by the inter- molecular
5   contacts and cross-links between the constituent enzym
molecules making up the crystal lattice, rather than by
the near-absence of water in the medium. As a result,
intermediate water concentrations can be tolerated by
enzymes when formulated as CLECs, as has previously n t
10 been possible (see Table 12). In commercial
applications, aqueous-organic solvent mixtures allow
manipulation of product formation by taking advantage
of relative solubilities of products and substrates.
Even in aqueous media, enzyme catalysts, immobilized or
15 soluble, are subject to mechanical forces within a
chemical reactor that can lead to denaturation and a
shortened half-life. The chemical cross-links within
the CLEC provide the necessary mechanical strength
(Quiocho and Richards, Proc. Natl. Acad. Sci. (USA) 52:
20 833-839 (1964)) that results in increased reactor life
for the enzyme catalyst.

A third advantage of a CLEC is that as a result of
its crystalline nature, a CLEC can achieve uniformity
across the entire cross-linked crystal volume.
25 Crystalline enzymes as described herein are grown and
cross-linked in an aqueous environment and, therefore,
the arrangement of molecules within the crystal lattice
remains uniform and regular. This uniformity is
maintained by the intermolecular contacts and chemical
30 cross-links between the enzyme molecules constituting

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the crystal lattice, even when exchanged into other
aqueous, organic or near-anhydrous media, or mixed
aqueous/organic solvents. In all of these solvents,
the enzyme molecules maintain a uniform distance fr m
5 each other, forming well-defined stable pores within
the CLECs that facilitate access of substrate to the
enzyme catalysts, as well as removal of product.
Uniformity of enzyme activity is critical in
industrial, medical and analytical applications where
10 reproducibility and consistency are paramount.
        A fourth advantage of using a CLEC is that it
should exhibit an increased operational and storage
half-life. Lattice interactions, even in the absence
of cross-linking, are known to stabilize proteins, due
15 in part to restrictions of the conformational degr es
of freedom needed for protein denaturation. In CLECs,
the lattice interactions, when fixed by chemical
cross-links, are particularly important in preventing
denaturation, especially in mixtures of aqueous and
20 non-aqueous solvents (Klibanov, A.M., Trends in
Biochemical Sciences 14: 141-144 (1989)). Enzymes that
have been in the crystalline state for months or years
routinely retain a high percentage of their catalytic
activity. Cross-linked immobilized enzyme crystals
25 stored in anhydrous solvents will be even further
protected from microbial contamination and damage,
which is a serious problem in storing large quantities
of protein in a nutrient rich, aqueous environment.
In the case of a lyophilized CLEC, the immobilized
30 enzyme is stored in the absence of solvent. That, and

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the stabilization achieved by cross-linking allows f r
the storage in the absence of refrigeration for long
periods of time.

    A fifth advantage of using a CLEC is that it
5 should exhibit enhanced temperature stability as a
consequence of the cross-links stabilizing the crystal
lattice. Carrying out reactions at a higher
temperature than that used with conventional methods
would increase reaction rates for the chemical
10 reactions of interest, both thermodynamically, and by
enhancing the diffusion rate into and out of the
crystal lattice of CLECs. These combined effects would
represent a major improvement in reaction efficiency,
because they would maximize the productivity of a given
15 quantity of enzyme catalyst, which is generally the
most expensive component of the reaction process
(Daniels, M.J., Methods in Enzymol. 136: 371-379
(1987)). The temperature stability exhibited by CLECs
is remarkable because most enzyme systems require mild
20 reaction conditions. CLECs would also be stabilized
against denaturation by transient high temperatures
during storage.

    A final advantage of use of a CLEC is that pores
of regular size and shape are created between
25 individual enzyme molecules in the underlying crystal
lattice. This restricted solvent accessibility greatly
enhances the metal ion or cofactor retention
characteristics of CLEC as compared to conventionally
immobilized enzymes and enzymes in solution. This
30 property of CLEC will permit the use of economically

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superior continuous-flow processes in situations (see
e.g. Oyama et. al. Methods in Enzymol. 136 503-516
(1987)) where enzyme would otherwise be inactivated by
metal ion or cofactor leaching. For example, in the
5 thermolysin-mediated synthesis of the dipeptidyl
aspartame precursor, Z-L-Asp-L-Phe-OMe, conventionally
immobilized enzyme is known to lose catalytic activity
in continuous-flow column processes, in part through
the leaching of calcium ions essential for thermolysin
10 activity. In practice, leaching of calcium ions has
forced the use of less efficient batch processes
(Nakanishi et. al., Biotechnology 3: 459-464 (1985)).
Leaching occurs when calcium ion complexes are formed
with substrate Z-L-Asp, in competition with the natural
15 calcium binding sites on the surface of the enzyme,
resulting in the loss of catalytic activity. The high
density of enzyme, and the correspondingly limited
volume accessible to solvent in the interstices of the
CLECs, discourages the formation of the competing
20 L-Asp-Ca^{++} complexes responsible for metal ion
leaching.

Preparation of CLECs - enzyme crystallization
     In the method of the present invention, a
cross-linked immobilized enzyme crystal (or CLEC) is
25 prepared as follows:
Enzyme crystals are grown by the controlled
precipitation of protein out of aqueous solution, or
aqueous solution containing organic solvents.
Conditions to be controlled include, for example, the

rate of evaporation of solvent, the presence of
appropriate co-solutes and buffers, and the pH and
temperature. A comprehensive review of the various
factors affecting the crystallization of proteins has
5   been published by McPherson (Methods Enzymol. 114: 112
(1985)). In addition, both McPherson and Gilliland (J.
Crystal Growth 90: 51-59 (1988)) have compiled
comprehensive lists of all proteins and nucleic acids
that have been reported as crystallized, as well as the
10   conditions that lead to their crystallization. A
compendium of crystals and crystallization recipes, as
well as a repository of coordinates of solved protein
and nucleic acid crystal structures, is maintained by
the Protein Data Bank (Bernstein et. al. J. Mol. Biol.
15   112: 535-542 (1977)) at the Brookhaven National
Laboratory. Such references can be used to determine
the conditions necessary for the crystallization of a
given protein or enzyme previously crystallized, as a
prelude to the formation of an appropriate CLEC, and
20   can guide the formulation of a crystallization strategy
for proteins that have not. Alternatively, an
intelligent trial and error search strategy (see eg.,
Carter, C.W. Jr. and Carter, C.W., J. Biol. Chem. 254:
12219-12223 (1979)) can, in most instances, produce
25   suitable crystallization conditions for most proteins,
including, but not limited to, those discussed above,
provided that an acceptable level of purity can been
achieved for these. The level of purity required can
vary widely from protein to protein. In the case of
30   lysozyme, for example, the enzyme has been crystallized

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directly from its unpurified source, the hen egg-white
(Gilliland, G.L., J. Crystal Growth 90: 51-59 (1988)).

     For use as CLECs in the method of this inventi n,
the large single crystals which are needed for X-ray
5   diffraction analysis are not required, and may, in
fact, be undesirable because of diffusion problems
related to crystal size. Microcrystalline showers (ie.
crystals of order 10^{-1} mm in size/cross section) ar
suitable for CLECs and are often observed, though
10 seldom reported in the X-ray crystallographic
literature. Micro-crystals are very useful in the
method of this invention to minimize problems with
diffusion (see eg., Quiocho, F.A., and Richards, F.M.,
Biochemistry 5: 4062-4076 (1967)).
15   In general, crystals are produced by combining the
protein to be crystallized with an appropriate aque us
solvent or aqueous solvent containing appropriate
precipitating agents, such as salts or organics. The
solvent is combined with the protein at a temperature
20 determined experimentally to be appropriate for the
induction of crystallization and acceptable for the
maintenance of protein stability and activity. The
solvent can optionally include co-solutes, such as
divalent cations, co-factors or chaotropes, as well as
25 buffer species to control pH. The need for co-solutes
and their concentrations are determined experimentally
to facilitate crystallization. In an industrial scale
process, the controlled precipitation leading to
crystallization can best be carried out by the simple
30 combination of protein, precipitant, co-solutes, and

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optionally buffers in a batch process. Alternative
laboratory crystallization methods, such as dialysis,
or vapor diffusion can also be adapted. McPherson
(Methods Enzymol. 114: 112 (1985)), and Gilliland (J.
5 Crystal Growth 90: 51-59 (1988)) include a
comprehensive list of suitable conditions in their
reviews of the crystallization literature.
Occasionally, incompatibility between the cross-linking
reagent and the crystallization medium might require
10 exchanging the crystals into a more suitable solvent
system.

Many of the proteins for which crystallization
conditions have already been described in the
literature, have considerable potential as practical
15 enzyme catalysts in industrial and laboratory chemical
processes, and are directly subject to formulation as
CLECs within the method of this invention. Table 1 is
a sampling of enzymes that have already been
crystallized. Note that the conditions reported in
20 most of these references have been optimized for the
growth of large, diffraction quality crystals, often at
great effort. Some degree of adjustment of conditions
for the smaller crystals used in making CLECS might be
necessary in some cases.

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Table 1

Enzyme	Microbial or biological source	References (including those cited therein)
• alcohol dehydrogenase	horse liver	Eklund *et al., J.Mol.Biol.* **146**: 561-587 (1981)
• alcohol oxidase	*Pichia pastoris*	Boys *et al., J.Mol.Biol.* **208**: 211-212 (1989) Tykarska *et al., J.Protein Chem.* **9**: 83-86 (1990)
• aldolase (fructose-bisphosphate)	rabbit muscle	Eagles *et al., J.Mol.Biol.* **45**: 533-544 (1969) Heidmer *et al., Science* **171**: 677-680 (1971)
	calf muscle	Goryunov *et al., Biofizika* **14**: 1116-1117 (1969)
	human muscle	Millar *et al., Trans.Roy.Soc.Lond.* **B293**: 209-214 (1981)
	Drosophila melanogaster	Brenner *et al., J..Biol.Chem.* **257**: 11747-11749 (1982)
• aldolase (PKDG)	*Pseudomonas putida*	Vandlen *et al., J.Biol.Chem.* **248**: 2251-2253 (1973)
• alkaline phosphatase	*Escherichia coli*	Sowadski *et al., J.Mol.Biol.* **150**: 245-272 (1981)
• asparaginase	*Erwinia carotova*	North *et al., Nature* **224**: 594-595 (1969)
	Escherichia coli	Epp *et al., Eur.J.Biochem.* **20**: 432-437 (1971)
	Escherichia coli	Yonei *et al., J.Mol.Biol.* **110**: 179-186 (1977)
	Proteus vulgaris	Tetsuya *et al., J.Biol.Chem.* **248**: 7620-7621 (1972)
• carbonic anhydrase	human erythrocyte (C)	Kannan *et al., J.Mol.Biol.* **12**: 740-760 (1965)
	human erythrocyte (B)	Kannan *et al., J.Mol.Biol.* **63**: 601-604 (1972)
	bovine erythrocyte	Carlsson *et al. J.Mol.Biol.* **80**: 373-375 (1973)
• catalase	horse erythrocyte	Glauser *et al., Acta Cryst.* **21**: 175-177 (1966)
	Micrococcus luteus	Marie *et al., J.Mol.Biol.* **129**: 675-676 (1979)
	Penicillium vitale	Vainshtein *et al., Acta Cryst.* **A37**: C29 (1981)
	bovine liver	Eventoff *et. al., J.Mol.Biol.* **103**: 799-801 (1976)
• creatine kinase	bovine heart	Gilliland *et al., J.Mol.Biol.* **170**: 791-793 (1983)
	rabbit muscle	McPherson, *J.Mol.Biol.* **81**: 83-86 (1973)
• glutaminase	*Actenobacter glutanimasificans*	Wlodawer *et al., J.Mol.Biol.* **99**: 295-299 (1975)
	Pseudomonas 7A	Wlodawer *et al., J.Mol.Biol.* **112**: 515-519 (1977)

Table 1 continued:

Enzyme	Microbial or biological source	References (including th se cited therein)
• glucose oxidase	*Aspergillus niger*	Kalisz *et al.*, *J.Mol.Biol.* 213: 207-209 (1990)
• ß-lactamases	*Staphylococcus aureus*	Moult *et al.*, *Biochem J.* 225: 167-176 (1985)
	Bacillus cereus	Sutton *et al.*, *Biochem. J.* 248: 181-188 (1987)
• lactate dehydrogenase	porcine	Hackert *et al.*, *J.Mol.Biol.* 78: 665-673 (1973)
	chicken	Pickles *et al.*, *J.Mol.Biol.* 9: 598-600 (1964)
	dogfish	Adams *et al.*, *J.Mol.Biol.* 41: 159-188 (1969)
	Bacillus stearothermophilus	Schar *et al.*, *J.Mol.Biol.* 154: 349-353 (1982)
• lipase	*Geotrichum candidum*	Hata *et al.*, *J.Biochem.* 86: 1821-1827 (1979)
	horse pancreatic	Lombardo *et al.*, *J.Mol.Biol.* 205: 259-261 (1989)
	Mucor meihei	Brady *et al.*, *Nature* 343: 767-770 (1990)
	human pancreatic	Winkler *et al.*, *Nature* 343: 771-774 (1990)
• luciferase	*Firefly*	Green, A.A., *et al.*, *Biochem. Biophys. Acta.* 20: 170 (1956)
• luciferase	*Vibrio harveyii*	Swanson *et al.*, *J.Biol.Chem.* 260: 1287-1289 (1985)
• nitrile hydratase	*Brevibacterium R312*	Nagasawa *et al.*, *Biochem.Biophys.Res. Commun.* 139: 1305-1312 (1986)
	P. chlororaphis B23	Nagasawa *et.al.*, *Eur.J.Biochem.* 162: 691-698 (1987)
• peroxidase	horseradish	Braithwaite *et al.*, *J.Mol.Biol.* 106: 229-230 (1976)
	horseradish roots (Type E4)	Aibara *et al.*, *J.Biochem.* 90: 489-496 (1981)
	Japanese radish	Morita, *Acta Cryst.* A28: S52 (1979)
• peroxidase (chloride)	*Caldaromyces fumago*	Rubin *et al.*, *J.Biol.Chem.* 257: 7768-7769 (1982)
• peroxidase (cytochrome)	*Sarchomyces cerevisae*	Poulos *et al.*, *J.Biol.Chem.* 253: 3730-3735 (1978)
• peroxidase (glutathione)	bovine erythrocyte	Ladenstein *et al.*, *J.Mol.Biol.* 104: 877-882 (1979)
• subtilisin	*Bacillus subtilis* (Novo)	Drenth *et al.*, *J.Mol.Biol.* 28: 543-544 (1967)
	Bacillus amyloliquefaciens (BPN')	Wright *et al.*, *Nature* 221: 235-242 (1969)
	Bacillus subtilis (Carlsberg)	Petsko *et al.*, *J.Mol.Biol.* 106: 453-456 (1976)

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Table 1 continued:

Enzyme	Microbial or biological source	References (including those cited therein)
• superoxide dismutase	bovine	Richardson et al., J.Biol.Chem. 247: 6368-6369 (1972)
	spinach	Morita et al., J.Mol.Biol. 86: 685-686 (1974)
	Saccharomyces cerevisiae, Escherichia coli	Beem et al., J.Mol.Biol. 105: 327-332 (1976)
	Bacillus stearothermophillus	Bridgen et al., J.Mol.Biol. 105: 333-335 (1976)
	Pseudomonas ovalis	Yamakura et al., J.Biol.Chem. 251: 4792-4793 (1976)
• thermolysin	Bacillus thermoproteolyticus	Matthews et al., Nature New Biol. 238: 37-41 (1972)
• urease	jack bean	Sumner,J.B., J.Biol. Chem. 69: 435 (1926)
• xylose isomerase	Streptomyces rubiginosus	Carrell et al., J.Biol.Chem. 259: 3230-3236 (1984)
	Arthrobacter B3728	Akins et al., Biochym.Biophys Acta 874: 375-377 (1986)
	Streptomyces olivochromogenes	Farber et al., Protein Engineering 1: 459-466 (1987)
	Streptomyces violaceoniger	Glasfeld et al., J.Biol.Chem. 263: 14612-14613 (1988)
	Actinoplanes missouriensis	Rey et al., Proteins:Struc.Func.Genet. 4: 165-172 (1988)

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Preparation of CLECs - cross-linking reaction

Once crystals are grown in a suitable medium, they
can be cross-linked. Cross-linking results in
stabilization of the crystal lattice by introducing
5   covalent links between the constituent enzyme molecules
in the crystal. This makes possible the transfer of
enzyme into an alternate reaction environment that
might otherwise be incompatible with the existence of
the crystal lattice, or even with the existence of
10 intact undenatured protein. Cross-linking can be
achieved by a wide variety of bifunctional reagents,
although in practice, simple, inexpensive
glutaraldehyde has become the reagent of choice. (For
a representative listing of other available
15 cross-linking reagents, one can consult, for example,
the 1990 catalog of the Pierce Chemical Company).

Cross-linking with glutaraldehyde forms strong
covalent bonds between primarily lysine amino acid
residues within and between the enzyme molecules in th
20 crystal lattice that constitute the crystal. The
cross-linking interactions prevent the constituent
enzyme molecules in the crystal from going back into
solution, effectively insolubilizing or immobilizing
the enzyme molecules into microcrystalline (ideally 10^{-1}
25 mm) particles. The macroscopic, immobilized,
insolubilized crystals can then be readily separated
from the feedstock containing product and unreacted
substrate by simple procedures such as filtration,
decantation, and others. They can also be used in CLEC
30 packed columns in continuous flow processes, where they

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exhibit enhanced cofactor and metal ion retention
properties.

By the method of this invention, CLECs are
obtained for use as enzyme catalysts in existing and
5   novel environments. The enhanced stability of the
CLECs, which results from the cross-linking reaction,
makes it possible to transfer the CLEC into a solvent
(e.g., aqueous, organic or near-anhydrous solvents, or
a mixture of these), in which it would otherwise be
10   incompatible, and to carry out chemical reactor
operation at elevated temperatures of extremes of pH.
The macroscopic CLEC catalyst particles can also be
readily manipulated, allowing recovery from feedstock
by simple methods, such as filtration, centrifugation,
15   or decantation of solvent. In addition, these can be
used in packed columns in continuous flow processes.

Preparation of CLECs - lyophilization

A suspension of one volume of cross-linked
thermolysin crystals in ten volumes of demineralized
20   water at pH 7.0 was lyophilized overnight using a
VirTis Model #24 lyophilizer. Lyophilized crystals
were stored at room temperature or at 4°C prior to
reconstitution, which was accomplished by adding ten
volumes of the solvent of choice directly onto crystals
25   taken from storage. Re-hydrated crystals were
reconstituted in 10mM calcium acetate buffer at pH 7.0
for the FAGLA cleavage experiments. Reconstituted
lyophilized CLECs were routinely stored at room
temperature. In contrast, soluble enzyme required

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storage at -70°C to maintain specific activity longer
than a week. This protocol was used for all the
enzymes described in the exemplification included here.

Synthesis of aspartame precursor with thermolysin CLEC

5   The method of the present invention, by which
cross-linked crystal enzymes are produced, is described
below and exemplified by the production of cross-linked
immobilized enzyme crystals of thermolysin for use in
the production of the dipeptidyl precursor of
10   aspartame, in ethyl acetate, which is a near-anhydrous
organic, solvent. Thermolysin, a protein which has
been crystallized and whose structure has been solved
at 1.6Å resolution (Holmes and Matthews, J. Mol. Biol.
160: 623-639 (1982)), is one example of an enzyme which
15   can be used as a CLEC in the present method.
Thermolysin is used in the manufacture of the
artificial sweetener aspartame (Isowa et. al. U.S.
Patent #4,436,925 (1984); Lindeberg, J. Chem. Ed. 64:
1062-1064 (1987); Nakanishi et. al., Biotechnology 3:
20   459-464 (1985); Oyama, et. al., Methods in Enzymol.
136: 503-516 (1987)). At the present time, most
aspartame appears to be produced by a conventional
synthetic chemistry approach, although use of
conventionally immobilized thermolysin in
25   near-anhydrous media has produced encouraging results
(Oyama et. al., J. Org. Chem. 46: 5242-5244 (1981);
Nakanishi et. al., Biotechnology 3: 459-464 (1985)).
Improvement in the enzymatic approach to aspartame
production, such as is possible through use of the

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present meth d, would make it competitive with the
presently-used method, both in terms of convenience and
cost (Oyama, et. al., Methods in Enzymol. 136: 503-516
(1987)).

5   Assessment of thermolysin CLECs

The method of the present invention has also been
used to produce thermolysin CLECs which have been
assessed as to their pH dependence and stability,
stability at elevated temperature, resistance to
10   exogenous proteolysis and stability in the presence of
an organic solvent. Thermolysin CLECs were compared t
soluble thermolysin, as described in detail in Exampl
2 and Figures 1-4. Results of the assessment showed the
following:
15   1.   As to pH dependence and stability, both
          forms demonstrate maximum activity at pH 7
          and demonstrate similar activity in the
          acidic range. In the alkaline pH range, the
          CLEC maintains maximum activity to pH 10; the
20   soluble thermolysin has 75% activity at pH
          8.5, only 25% activity at pH 9 and is
          completely inactive at pH 9.5.
          2.   The additional stabilization achieved in
          CLECs results in enzymatic activity at higher
25   temperatures than is possible with soluble
          thermolysin. Enhanced stability of CLEC
          thermolysin at lower temperatures makes
          storage simpler than it is for the soluble
          enzyme. Thermal stability and resistance to

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autolysis was also dem nstrated f r
thermolysin CLECs, which retained maximum
activity after five days of incubation at
65°C. In contrast, soluble thermolysin lost

5   50% of its initial activity after two hours
incubation and demonstrated negligible
activity after 24 hours incubation at 65°C.
3.   Enzymatic activity of thermolysin CLECs
was unaffected by four days' incubation in

10   the presence of the powerful streptococcal
protease, Pronase®. In contrast, soluble
thermolysin was rapidly degraded and lost all
activity after 90 minutes incubation.
4.   Thermolysin CLECs and soluble

15   thermolysin exhibited markedly different
stability in the presence of organic
solvents, as shown in Table 12. The
thermolysin CLECs retained greater than 95%
maximum activity following incubation with

20   all organic solvents assessed.
These features of thermolysin CLECs and other
enzyme CLECs make them particularly useful, since they
are easier to store, more stable and less easily
inactivated or degraded than corresponding soluble

25   enzymes.

Assessment of Elastase CLECs

The method of the present invention has also been
used to produce elastase CLECs which have been assessed
as to their activity and resistance to exogenous

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proteolysis. Elastase CLECs were compared to soluble
elastase, as described in detail in Example 3 and
Figures 5 and 6. Results of the assessment
demonstrated the following:

5   1.   Elastase CLECs retain approximately 50%
activity compared to soluble enzyme.

       2.   Soluble elastase was rapidly degraded by
protease. Activity of soluble elastase was
reduced to 50% of initial activity following

10   ten minutes incubation in the presence of
protease. After one hour incubation the
soluble enzyme had lost more than 90% of its
initial activity. In contrast the enzymatic
activity of the elastase CLEC was unaffected

15   by incubation with protease.

Assessment of Esterase CLECs

     The method of the present invention has also been
used to produce esterase CLECs which have been assessed
as to their activity and resistance to exogenous

20 proteolysis. Esterase CLECs were compared to soluble
esterase, as described in detail in Example 4 and
Figures 7 and 8. Results of the assessment
demonstrated the following:

       1.   Esterase CLECs retain approximately 50%

25   activity compared to soluble enzyme.

       2.   Soluble esterase was highly susceptible to
proteolytic degradation. Activity of soluble
esterase was reduced to 50% of initial
activity following ten minutes incubation in

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the presence of protease. After one hour
incubation the soluble enzyme had lost more
than 90% of its initial activity. In
contrast the enzymatic activity of the
5   esterase CLEC was unaffected by incubation
with protease.

Assessment of Lipase CLECs

        The method of the present invention has also b en
used to produce lipase CLECs which have been assessed
10 as to their activity. Lipase CLECs were compared to
soluble lipase, as described in detail in Example 5 and
Figure 9. Results of the assessment demonstrated that
lipase CLECs retain approximately 90% activity compared
to soluble enzyme.

15 Assessment of Lysozyme CLECs

        The method of the present invention has also been
used to produce lysozyme CLECs which have been assessed
as to their activity and resistance to exogenous
proteolysis. Lysozyme CLECs were compared to soluble
20 lysozyme as described in detail in Example 6 and Figure
10. Results of the assessment demonstrated that
lysozyme CLECs retain approximately 50% activity
compared to soluble enzyme.

Assessment of Asparaginase CLECs

25   The method of the present invention has also been
used to produce asparaginase CLECs which have been
assessed as to their activity. Asparaginase CLECs were

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compared to soluble asparaginase, as described in
detail in Example 7 and Figure 11. Results of the
assessment demonstrated the following asparaginase
CLECs retain approximately 77% activity compared t
5 soluble enzyme.

General applicability of CLECs

As disclosed here, CLECs represent a novel
technology with broad use in many areas, including, but
not limited to, industrial scale syntheses, laboratory
10 tools, biosensors, and medical applications. Examples
of various systems using conventionally immobilized
enzyme methods in their execution are given in Tables
2-5 below. One skilled in the art should be able to
adapt these, and similar systems, to the CLEC
15 technology disclosed in this application. To
illustrate this, specific examples are discussed in
more detail from each of the categories listed.

Table 2 below lists examples which use
conventionally immobilized enzymes in an industrial
20 process, which examples can be readily adapted to the
CLEC technology disclosed here.

Table 2

Enzyme	Producti n or application	Substrates	References (including those cited)
• thermolysin	• aspartame precursor	Z-Asp, L-Phe-OMe	Oyama *et al.*, *J. Org. Chem.* 46: 5242-5244 (1981) Nakanishi *et al.*, *Trends in Biotechnology* 3: 459-464 (1985)
• subtilisin	• aspartame	L-Asp-L-Phe, OMe	Davino,A.A., US Patent 4293648 (1981)
• lipase	• cocoa fat substitutes	palm oils	Harwood, J., *Trends in Biochemical Sciences* 14: 125-126 (1989) Macrae, A.R., *J. Am. Oil Chem Soc.* 60: 291-294 (1983)
• nitrile hydratase, nitrilases, amidase	• acrylamide	acrylonitrile	Nagasawa, T. and Yamada, H., *Trends in Biotechnology* 7: 153-158 (1989)
• amino acylase (fungal) • amino acid esterase, • subtilisin • amidases • hydantionases • specific dehydrogenases • amino peptidase • transaminase	• amino acid resolution	N-acyl-D,L amino acids esters of D,L amino acids amides of D,L amino acids hydantoins a-hydroxycarboxylic acids	Schmidt-Kastner,G. & Egerer, P. in *Biotechnology* vol 6a: 387-421 (1984) and references therein. Fusee,M.C., *Methods in Enzymology* 136: 463 (1987)
• amino acid dehydrogenase + formate dehydrogenase	• amino acid production: general	keto or hydroxy acids	Fusee,M.C., *Methods in Enzymology* 136: 463 (1987) Rozzell,J.D., *Methods in Enzymology* 136: 479 (1987)
	• amino acid production: specific		Enzymes in Industry: Ed Gerhartz.W.,VCH Press 1990
• L-aspartase • L-aspartate 4-decarboxylase aspartase + L aspartate 4 decar-boxylase • ACL hydrolase • L-ACT hydrolase	L aspartic acid L-alanine L-lysine L-cysteine L-isoleucine L-methionine	fumarate/fumaric acid L-aspartic acid, ammonium fumarate D,L-a amino e-caprolactam (ACL) DL-2amino2 thiazoline 4carboxylic acid	
• lyase • L-tryptophan synthetase • fumarase • hydantoinase	L-phenylalanine L-tryptophan L-valine L-malic acid D n carbamoyl p-hydroxy- phenyl glycine	cinnamate indole, L-serine fumarate 5p-hydroxy hydantoin	

Table 2 continued:

Enzyme	Production or application	Substrates	References (including those cited)
• lipases, esterases	• resolution of racemates by stereoselective synthesis	synthetic chemistry	Jones, J.B., *Tetrahedron* 42: 3351-3403 (1988) Butt, S. and Roberts, S.M., *Natural Product Reports* 489-503 (1986), and references cited therein for a more comprehensive review of this area
• fumarase	• L-malic acid	fumaric acid	Chibata *et al.*, *Methods in Enzymology* 136: 455 (1987)
• lactase, ß-galactosidases	• disaccharide synthesis eg galactosyl-N- acetyl galactosamine	lactose & N-acetyl galactosamine	Larsson *et al.*, *Methods in Enzymology* 136: 230 (1987)
• lipase, esterase	• L-menthol	4 isomer mix	Fukui,S., Tanaka,A., *Methods in Enzymology* 136: 293 (1987)
• amidases	• D-valine (intermediate for pyrethroid insecticide fluvinate)	D,L amino acid amide	Schmidt-Kastner,G. & Egerer, P. in *Biotechnology* vol 6a: 387-421 (1984) and references therein.
• lipase (Candida cylindricea)	• R(+)2 phenoxy-propionic acids (herbicides)	2 chloro propionic acids	*Biocatalysts in Organic Syntheses* eds Tramper, van de Plas & Linko: Proceedings of International Symposium in Netherlands 1985
• lipases, esterases, amidases, aldolases • proteases, peptidases • yeast lipase	• organic syntheses monoglycerides peptides • 2(p-chlorophenoxy) propionic acid: herbicide	resolution of racemic ester	Jones, J.B., *Tetrahedron* 42: 3351-3403 (1988) Butt, S. and Roberts, S.M., *Natural Product Reports* 489-503 (1986), and references cited therein for a more comprehensive review of this area
• strictodine synthetase	• alkaloid production eg strictosidine		Pfitzner *et al.*, *Methods in Enzymology* 136: 342 (1987)
• penicillin acylase • penicillin amidase	• 6-amino penicillinanic acid and 7-ADCA	penicillin G or V	*Enz Eng* 6: 291 (1982) *Enz Eng* 8: 155
• hydroxysteroid dehydrogenases	• steroid transformations		Carrea *et al*, *Methods in Enzymology* 136: 150 (1987)
• 5'phosphodiesterase, nucleases	• 5'-ribonucleotides		Keller *et al*, *Methods in Enzymology* 136: 517 (1987)
• esterase	• ß-lactam precursor (chiral mono esters eg ßamino glutaric acid monoalkyl ester)	corresponding diesters	Japanese patent application: 82-159, 493 (1981) Biseibutsu Company
• lipases	• ß-blockers		Kloosterman,M *et al.*,*Trends in Biotechnology* 6: 251-256 (1988)

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<u>Production of acrylamide using CLEC technology</u>

The following is a description of one use of the
method of the present invention: the adaptation of
acrylamide production from immobilized cells which
5   overproduce nitrile hydratase enzyme (Nagasawa, T. and
Yamada, H., Trends in Biotechnology 7: 153-158 (1989))
to the CLEC technology previously disclosed herein.

Industrial scale production of acrylamide, an
important commodity chemical, has been described by
10 Yamada and collaborators (Nagasawa, T. and Yamada, H.,
Trends in Biotechnology 7: 153-158 (1989)). Kilotons
of acrylamide per year are produced in chemical
reactors loaded with entrapped cells selected as
overproducers of the enzyme nitrile hydratase. Nitrile
15 hydratase has also been reported as purified and
crystallized from two sources, <u>Brevibacterium</u> R312
(Nagasawa et. al., Biochem. Biophys. Res. Commun. 139:
1305-1312 (1986) and <u>P. chlororaphis</u> B23 (Nagasawa et.
al., Eur. J. Biochem. 162: 691-698 (1987). As
20 disclosed here, these crystalline enzymes can each be
immobilized by crosslinking with glutaraldehyde or
other suitable crosslinking reagent to produce a CLEC.
The nitrile hydratase CLECs can then be used in a
conventional reactor, in place of the entrapped cells
25 currently used. Adapting this process to CLEC
technology leads to immediate advantages. These
include; reduced plant size and improved throughput
resulting from the enhanced activity per unit volume
implicit in the higher enzyme concentration in the
30 CLECs, and improved substrate and product diffusion

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rates; reduction in undesired contamination and side
reactions, resulting from the higher purity of CLECs;
and reduced sensitivity to microbial contamination in
the absence of cells. In addition, there are other
5 benefits available only to a CLEC-based method. These
benefits include: higher temperature operation to
improve reaction rates; the ability to operate in
aqueous, organic and near-anhydrous solvents, allowing
optimization of the acrylamide production reaction;
10 and enhanced half-life in operation and storage,
resulting from the higher chemical and mechanical
stability of CLECs, particularly in unconventional
solvents.

Medical applications of CLEC technology -
15 extracorporeal treatment
       The method of the present invention and an·
appropriately selected CLEC or set of CLECs can also be
used for medical applications. A CLEC or a set of
CLECs can be used, for example, to remove a component
20 of a fluid, such as blood, generally by altering the
component, and thus, converting it to a substance not
detrimental to an individual or which can be removed by
normal body processes (e.g., via detoxification, or
degradation in the liver, excretion via the kidneys).
25 In this application, an appropriately-selected CLEC or
set of CLECs is brought into contact with body fluid,
which contains the component to be altered, or a
reactant (product or substrate) of a reaction in which
the component participates, upon which the enzyme in

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the CLEC acts. As a result, the enzyme is able to act upon the component to be altered or with another substance which is a product of a reaction in which the component to be altered participates. The activity of

5   the enzyme results in direct alteration of the component to be removed or in alteration of the product of the reaction in which the component participates (thus making continuation of the reaction impossible). This can be carried out through the use of an

10 extracorporeal device which includes an appropriately-selected CLEC or set of CLECs and a retaining means which is made of a material; such as a porous material on which a CLEC is retained or a tube in which a CLEC is present, which allows contact

15 between the component itself or the substance in the fluid which is a product of a reaction in which the component to be altered participates.

        This might also be achieved by the insertion of an appropriate CLEC into a suitable body compartment, such

20 as the peritoneum or a lymph node, where the CLEC would have access to bodily fluids. This insertion might be done surgically, or by injection of the CLEC slurry. Direct injection of CLEC into the blood stream would not be appropriate, given the high risk of embolism.

25   The use of appropriate CLECs in this area might serve as an alternative to genetic methods in enzyme replacement therapy to correct a natural deficiency, such as, e.g. phenylketonuria.

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Table 3 illustrates some of the medical applications in which CLECs could be used. For the majority of these cases, the extra-corporeal treatment is still in the research phase, but the benefits that
5 CLECs offer could provide novel treatments in areas in which there was previously no alternative treatment.

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TABLE 3

Enzyme employed	Removal of:-	Disease/patients treated	References
• asparaginase	• asparagine	• leukemia (Removal of asparagine, an important cancer nutrient, harms leukemic cells which cannot manufacture the essential amino acid - asparagine; normal cells can manufacture asparagine and so are unaffected by this treatment.)	Klein,M., Langer,R., *Trends in Biotechnology* 4: 179-185 (1986) and references therein Chang,T.M.S., *Methods in Enzymology* 137: 444-457 (1987) & references therein
• heparinase	• heparin	• deheparinization for hemoperfusion patients eg kidney dialysis	Langer,R.,*et al., Science* 217: 261-263 (1982)
• bilirubin oxidase	• bilirubin	• neo-natal jaundice	Lavin,A.,*et al., Science* 230: 543-545 (1985)
• carboxypeptidase	• methotrexate	• chemotherapy patients	Pitt,A.M.,*et al., Appl. Biochem.Biotechnol.*8: 55-68 (1983)
• tyrosinase	• aromatic amino acids	• liver failure exhibiting pathological elevations of amino acids	Chang,T.M.S., *Sem.Liver Dis.Ser.* 6: 148 (1986)
• phenylalanine ammonium lyase	• phenylalanine	• phenylketonuria and liver failure	Ambrus,C.M., *et al., J.Pharm.& Exp.Ther.* 224: 598-602 (1983)
• multi-enzyme system including: urease, glutamate dehydro-genase, glucose, dehydrogenase & a transaminase	• urea (converted into glutamic and other amino acids, via ammonia)	• detoxification for chronic renal failure patients	Chang,T.M.S., *Methods in Enzymology* 137: 444-457 (1987) & references therein Chang,T.M.S., *Enzyme Eng* 5: 225 (1980)
• arginase	• arginine	• familial hyperargininaemia	Kanalas,J.J. *et al., Biochem. Med.* 27: 46-55 (1982)
• glutamate dehydrogenase & ammonia	• ammonia	• liver failure	Maugh,T.H., *Science* 223: 474-476 (1984)

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A particular application of the present method is the heparin lyase system for blood deheparinization (Bernstein et al., Methods in Enzymology 137: 515-529 (1987)), which is discussed below.

5   All extracorporeal devices perfused with blood, such as kidney dialysis, continuous arteriovenous hemofiltration or extracorporeal membrane oxygenators, require heparinization of the patient to avoid blood clotting. However, heparinization of the patient leads

10   to hemorrhagic complications and remains a threat to human safety. These problems increase as the perfusi n times increase, for example with the membrane oxygenator, and can lead to serious bleeding. After extracorporeal therapy, heparin may be removed from

15   blood by employing a heparinase device at the effluent of the extracorporeal device which eliminates all heparin from the blood returned to the patient and thus avoids the current problems of heparinization.

Published research (Langer et al. Science 217:

20   261-263 (1982)); Bernstein et al., Methods in Enzymology 137: 515-529 (1987)), details the problems presented by conventionally immobilized enzymes used in extracorporeal devices. The principal problem is that conventional immobilization results in a low retenti n

25   of enzyme activity per unit volume, thus requiring a large volume of immobilized enzyme to perform the necessary heparinization. This volume is too large t be of practical use in humans. However, the high retention of activity per unit volume in CLEC, due to

30   the lack of inert support circumvents this problem and

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offers a practical solution t deheparinization of
humans. The enhanced stability of CLEC will reduce the
disassociation of enzyme from the crosslinked crystal.
This is superior to the less stable, conventionally
5   immobilized enzymes, because immune responses resulting
from enzyme leakage will be reduced. CLEC temperature
stability prevents denaturation of the enzyme due to
high transient temperatures during storage; it is
likely that CLEC may retain high activity, even when
10 stored at room temperature. In addition, CLEC will be
cheaper and more convenient to use than their
conventionally immobilized counterparts because of
their longer operational and storage lifetimes.

Additional applications of CLEC technology: biosensors
15   A CLEC or a set of CLECs can be used as a
component of a sensor, referred to as a biosensor,
useful for detecting and/or quantitating an analyte f
interest in a fluid, such as body fluid (e.g., blood,
urine), chemical and laboratory reaction media, organic
20 media, water, culture medium and beverages. In some
instances, the fluid in question can be a gas, as in an
alcohol breath analyzer (Barzana, E., Klibanov, A., and
Karell, M., NASA Tech Briefs 13:104 (1989)). In this
application an appropriately-selected CLEC or set of
25 CLECs is brought into contact with a fluid to be
analyzed for the analyte of interest. The analyte of
interest can be measured directly (e.g., blood glucose
level) or indirectly (e.g., by detecting or
quantitating a substance which is a reactant (product

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or substrate) in a reaction in which the analyte of
interest participates). In either case, the CLEC is
able to act upon the analyte or the substance which is
a reactant in a reaction in which the analyte also
5   participates. The activity of the enzyme results in a
detectable change (e.g., change in pH, production of
light, heat, change in electrical potential) which is
detected and/or quantitated by an appropriate detecting
means (e.g., pH electrode, light or heat sensing
10   device, means for measuring electrical change) (Janata,
J., et al., Anal. Chem. 62: 33R-44R (1990)). Any means
useful for detecting the change resulting from the
enzyme-catalyzed method can be used. A biosensor of
the present invention includes a CLEC or set of CLECs
15   and a retaining means for the CLEC which allows contact
between the CLEC(s) and the analyte of interest or th
substance in the fluid which is a reactant in the
reaction in which the analyte of interest participates.
        Table 4 illustrates some of the biosensor
20   applications in which CLECs could be used. Currently
immobilized enzymes are used in these applications, but
suffer from low stability, low enzyme density, short
lifetimes and lack of reproducibility. These examples
can be readily adapted to the CLEC technology disclosed
25   here.

Table 4

Enzyme Employed	Detection of:-	Application	Reference
• glucose oxidase	• glucose	• diabetics	• Daniles,B.,Mossbach,K., *Methods in Enzymology* 137: 4-7 (1987) • Hall,E *"Biosensors "* Open University Press (1990) • Taylor,R., *Proceed. Biotechnology Conference* 1989; 275-287 • Anthony *et al.,* *"Biosensors, Fundamentals and Applications"*, Oxford University Press (1987)
• creatinine deiminase	• creatinine	• kidney function	• Tabata,M. *et al., Anal. Biochem.* 134: 44 (1983)
• urease	• urea	• kidney function	• Hsuie,G.H *et al., Polym.Mater.Sci.Eng.* 57: 825-829 (1987) • Kobos, *et al. Anal.Chem.* 60: 1996-1998 (1988)
• lactate oxidase & dehydrogenase	• lactate	• clinical applications	• Blaedel, W.J. & Jenkins,R. A., *Anal. Chem.* 48(8): 1240 (1976) • Sagaguchi, Y.,*et al., J. Appl. Biochem* 3: 32 (1981)
• glucose-6-pyruvate dehydrogenase	• Glucose-6-phosphate, sucrose and ATP	• diabetics and other medical	• ibid as glucose oxidase
• alcohol dehydrogenase, alcohol oxidase	• ethanol & other alcohols; acetic, formic acids	• breathalysers and industrial applications	• Romette,J.L.*et al., Methods in Enzymology* 137: 217-225 (1987) • Ho,M.H., *Methods in Enzymology* 137: 271-288 (1987)
• ß-fructosidase	• sucrose	• industrial applications	•.Romette,J.L.*et al., Methods in Enzymology* 137: 217-225 (1987)
• cholesterol oxidase	• cholesterol	• cholesterol testing	• Satoh,I.*Methods in Enzymology* 137: 217-225 (1987)
• catalase	• uric acid, cholesterol	• atherosclerotic and other medical	• Satoh,I.*Methods in Enzymology* 137: 217-225 (1987)
• carboxy peptidase	• methotrexate	• cancer	• ibid as glucose oxidase
• carbonic anhydrase	• carbon dioxide	• industrial, laboratory & environmental applications	• ibid as glucose oxidase
• L-amino acid oxidase	• amino acids	• medical and industrial	• ibid as glucose oxidase
• ß-lactamase penicillinase	• penicillin	• medical	• Anzai *et al., Bull.Chem. Soc.Jpn.* 60: 4133-4137 (1988)
• alkaline phosphatase	• phosphate	• metabolite monitoring	• ibid as glucose oxidase
• nitrate/nitrite reductase	• nitrates & nitrites	• metabolite and food monitoring	• ibid as glucose oxidase

Table 4 continued:

Enzyme Employed	Detection of:-	Application	Reference
• arylsulfatase	• sulfate	• metabolite monitoring	• ibid as glucose oxidase
• succinate dehydrogenase	• succinate	• industrial	• ibid as glucose oxidase
• bacterial luciferase	$FMNH_2$ and coupled reactions	• detection of 10^{-18} molar quantities of $FMNH_2$ through measurement of photon release	• WannlundJ.,et al., "Luminescent assays: Perspectives in endocrinology and clinical chemistry"; Eds Serio.M. and Pazzagli,M. 1: 125 (1982) • Kurkijarvi et al., Methods in Enzymology 137: 171-181 (1987)
• firefly luciferase	ATP and coupled reactions	• detection of 10^{-12} molar quantities of ATP through measurement of photon release	• Kurkijarvi et al., Methods in Enzymology 137: 171-181 (1987) • Murachi et al., Methods in Enzymology 137: 260-271 (1988)

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In the method of the present invention as it is carried out for the analysis of samples in a biosens r, it is particularly desirable to produce the largest possible detectable signal from the smallest possible
5   quantity of substrate and catalyst. In this regard, the CLEC technology disclosed here is particularly attractive, since it achieves the highest possible concentration of enzyme catalyst in a given volume.

Often, considerable efforts are made to couple an
10 ultimate enzymatic reaction of interest, either directly, or through suitable intermediates, to the production of light by enzymes like luciferase (Kurkijarvi et al., Methods in Enzymol. 137: 171-181 (1988)). This is done in order to take advantage of
15 the unparalleled sensitivity and efficiency of photon detection equipment, which allows for the detection of femtomolar concentrations of enzyme reaction products under appropriate conditions. Following this principle, biosensor systems have been designed, using
20 conventionally immobilized enzymes, to detect various substrates of clinical and other interest. Light producing reactions have been coupled to assay reactions detecting substrates like, D-glucose, L-lactate, L-glutamate and ethanol, among others, at
25 extremely low concentration.

With regard to this application, the luciferase enzyme from Vibrio harveyii has been reported as crystallized (Swanson et al., J. Biol. Chem. 260: 1287-1289 (1985)). Crystals of this luciferase can be
30 crosslinked with glutaraldehyde or other suitable

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reagent to form a CLEC of luciferase. For biosens r
and analytical uses, a CLEC of luciferase offers many
advantages over conventionally immobilized enzyme. In
a CLEC, the entire volume of the luciferase CLEC w uld
5 consist of light emitting enzyme. In a conventionally
immobilized enzyme system, however, as much as 95% of
the total volume is taken up by "inert" carrier
material, which more likely functions as an absorber of
the light emitted by enzyme. In addition, the enhanced
10 stability of CLECs should facilitate storage at ro m
temperature, and also makes possible novel sensing
applications in harsh environments and elevated
temperatures.

Additional applications of CLEC technology - laborat ry
15 reactions

      CLECs may be used as laboratory reagents in small
columns or in batch processes, which can be used to
carry out laboratory reactions. Some of the broad
categories of reactions are in Table 5.

Table 5

Enzyme Employed	Reaction type catalyzed	Reference
• lipases, phospholipases	• Stereoselective synthesis: including esterification, transesterification, aminolysis, lactonizations, polycondensations, acylation, oximolysis and resolution of racemic mixtures	• Zaks,A.& Klibanov,A.M. *Proc.Nat.Acad.Sci.USA.* 82: 3192-3196 (1985) • Klibanov,A.M. *Acc.Chem.Res.* 23: 114-120 (1990) and references therein • Wong,C.H.,*Chemtracts-Organic Chemistry* 3:91-111 (1990) and references therein
• esterases	• stereoselective synthesis and resolution	• Kobayashi et al., *Tetrahedron Letters* Vol 25, #24: 2557-2560 (1984) • Schneider et al., *Agnew. Chem. Int. Ed.Engl.* 23 (#1): 64-68 (1984)
• tyrosinase	• oxidation of phenols to produce quinones	• Kazandjian,R.Z and Klibanov, A.M. *J.Am.Chem.Soc* 110: 584-589 (1986)
• proteases, eg subtilisin	• stereoselective acylation of carbohydrates	• Riva et al. *J.Am.Chem.Soc.* 110: 584-589 (1988)
• oxidases	• selective oxidation of hydrocarbons	• Klibanov,A.M. *Acc.Chem.Res.* 23: 114-120 (1990) and references therein
• other enzymes not requiring co-factors: isomerases, lyases, aldolases, glycosyl transferases, glycosidases	• Stereoselective synthesis:	• Wong,C.H.,*Chemtracts-Organic Chemistry* 3:91-111 (1990) and references therein
• other enzymes not requiring added co-factors: flavoenzymes, pyridoxal phosphate enzymes, metalloenzymes	• Stereoselective synthesis:	• Wong,C.H.,*Chemtracts-Organic Chemistry* 3:91-111 (1990) and references therein
• enzymes requiring co-factors: kinases (ATP), oxidoreductatses (NAD/P), methyl transferases (SAM), CoA-requiring enzymes, sulfurases (PAPS)	• Stereoselective synthesis:	• Wong,C.H.,*Chemtracts-Organic Chemistry* 3:91-111 (1990) and references therein

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.Schneider et al. (Agnew. Chem. Int. Ed. Engl. 23
(No.1): 64-68 (1984)) illustrates how enzymes may be
used in organic syntheses. Pig liver esterase was
employed in the meso-ester transformation into a chiral
5   mono-ester in an aqueous phosphate buffer.

The advantages of CLEC catalyzed reactions for
laboratory use are threefold. First, CLEC retain high
activity in harsh environments (eg. aqueous, organic,
near-anhydrous solvents and mixtures of these, and at
10 high temperatures) that are typical of laboratory
chemical synthesis experiments. Second, CLEC exhibit
high operational and storage stability, which is
appropriate for intermittent laboratory experiments.
Third, their high activity per unit volume will allow.
15 shorter reaction times and require smaller volumes of
enzyme (per unit of activity). Thus, the advantages
that CLECs offer over free or immobilized enzymes,
provide organic chemists with an alternative, highly
selective, synthetic tool.

20   In all of these instances described above, but n t
limited to these, the method of this invention can b
adapted by one of ordinary skill in the art, to convert
a process using a conventionally immobilized enzym
catalyst to the use of a CLEC of the appropriate
25 enzyme. CLECs can not only replace conventional
immobilized enzymes, but also be used in cell mediated
transformations. The present invention will now be
illustrated by the following Examples which are not
intended to be limiting in any way.

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EXAMPLE 1

<u>Crystallization and crosslinking of thermolysin for</u>
<u>synthesis of the aspartame precursor, Z-Asp-Phe-OMe.</u>
     <u>Crystallization</u>

5   250 mg of thermolysin from <u>Bacillus</u>
<u>thermoproteolyticus</u> was purchased from
Boehringer-Mannheim GmbH, and dissolved in 4 ml of 45%
dimethyl sulfoxide (DMSO) and 55% 1.40 M calcium
acetate, 0.50 M sodium cacodylate at pH 6.5. These

10   starting conditions are similar to those described by
Matthews et. al. for the production of diffraction
quality thermolysin crystals (see, eg., Holmes and
Matthews, J. Mol. Biol. 160: 623-639 (1982)). The
protein solution was then concentrated to 1 ml in a

15   Centricon 10 micro-concentrator.   A good yield of
microcrystals was obtained by a process of flash
crystallization, now disclosed here, in which 1 ml of
water, or 1.40 M calcium acetate, 0.50 M sodium
cacodylate at pH 6.5, was rapidly injected into either

20   of the thermolysin-DMSO solutions described above. A
shower of hexagonal micro-crystals of approximately
uniform dimensions (approx. 10^{-1} mm in length) results
from this process.

     <u>Crosslinking of thermolysin microcrystals.</u>

25   The protocol used in this specific example of the
method of this invention is an adaptation of that
described by Nakanishi et. al. (Biotechnology 3:
459-464 (1985)), in which protocol, thermolysin was
first adsorbed onto a carrier bead composed of the

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ion-exchange resin Amberlite XAD-7, and subsequently
immobilized by cross-linking with glutaraldehyde
(Quicho and Richards, Proc. Natl. Acad. Sci. (USA)
52:833-839 (1964)). In this exemplification, the
5 microcrystals of thermolysin obtained above were
centrifuged and pelleted, and the supernatant was
discarded. 5 ml of 17.5% technical grade
glutaraldehyde, in 2.5 % DMSO, 0.05M calcium acetate,
and 0.025M sodium cacodylate at pH 6.5, were then added
10 to the microcrystals. The mixture was incubated with
gentle agitation at 37°C for 4 hours. The crosslinking
reaction was stopped by repeated washing of the
crystals with 10 ml aliquots of water to remove the
glutaraldehyde solution. The washed cross-linked
15 thermolysin crystals constitute the thermolysin CLEC
used below as a catalyst.

Synthesis of Z-Asp-Phe-OMe in an aqueous solution
        5 ml of a thermolysin CLEC suspension were added
to a continuous stirred-batch reactor incubated at
20 37°C. After centrifugation and decantation of the
supernatant, an aqueous reaction mixture was added to
the CLECs. This solution was prepared by mixing 80 mg
of Z-L-Asp and 80 mg of L-Phe-OMe-HCl in 1 ml of water,
with acetic acid added to obtain a pH of 7.0. Samples
25 were taken for analysis by HPLC. Table 6 below sh ws
the HPLC peak height of the Z-L-Asp substrate peak
after the indicated time of reaction, normalized to 1
at time t=0. Since Z-L-Asp is rate limiting in this
reaction, measuring its depletion is equivalent to

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measuring the appearance f product Z-L-Asp-L-Phe-OMe
(Nakanishi et. al. Biotechnology 3: 459-464 (1985)).
Table 6 also includes the normalized peak height of
limiting Z-L-Asp substrate remaining, and an estimate
of the degree of completion of the reaction. It is
clear that the reaction proceeded to about 20%
completion within the first 30 seconds and plateaued
there. These results are consistent with the
observations of Nakanishi et al. (Biotechnology 3:
459-464 (1985)) when using conventionally immobilized
thermolysin in an aqueous reaction mixture as above,
and are attributable to the sparing solubility of the
Z-L-Asp-L-Phe-OMe product in water.

TABLE 6

Reaction Time (sec)	Peak Height (Normalized)	Percent Completion
0	1.000	
30	0.727	27.3%
60	0.857	14.3%
120	0.940	6.0%
180	0.797	20.3%

Synthesis of Z-Asp-Phe-OMe in a near-anhydrous solution

5 ml of a thermolysin CLEC suspension were added
to a continuous stirred-batch reactor incubated at
37°C. After centrifugation and decantation of the
supernatant, a near-anhydrous organic reaction mixtur
was added to the CLECs. This solution was prepared by
mixing 80 mg of Z-L-Asp and 240 mg of L-Phe-OMe in 1 ml

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of 99% ethyl acetate and 1% water. Samples were tak n
for analysis by HPLC. Table 7 below shows the HPLC
peak height of the Z-L-Asp substrate peak after the
indicated time of reaction, normalized to 1 at time

5   t=0. Since Z-L-Asp is rate limiting in this reacti n,
measuring its depletion is equivalent to measuring the
appearance of product Z-L-Asp-L-Phe-OMe (Nakanishi et.
al. Biotechnology 3: 459-464 (1985)). Table #7 also
includes the normalized peak height of limiting Z-L-Asp

10 substrate remaining, and an estimate of the degree f
completion of the reaction. In this case, the reacti n
proceeded to about 70% completion within the first 30
seconds and plateaued there. These results are also
consistent with the observations of Nakanishi et al.

15 (Biotechnology 3: 459-464 (1985)) with conventionally
immobilized thermolysin in a near-anhydrous reaction
mixture, and are attributable to product inhibition of
the enzyme.

TABLE 7

20	Reaction Time (sec)	Peak Height (Normalized)	Percent Completion
	0	1.000	
	30	0.323	67.7%
	60	0.314	68.6%
25	120	0.305	69.5%
	180	0.272	72.8%

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EXAMPLE 2
Crystallization, cross-linking and lyophilization of
thermolysin and assessment of characteristics of
resulting product

5   Crystallization of thermolysin
        Thermolysin (Diawa Kasei K.K., Japan) was
dissolved in 10 mM calcium acetate (Sigma), pH 10.0, to
a concentration of 10% (w/v). The pH of the solution
was maintained at 10.0 by titration with 2 M NaOH.
10 Following complete solubilization, the protein solution
was titrated to pH 8.0 with 2 M HCl. Solid calcium
acetate was added to 1.2 M. Dimethyl sulfoxide (Sigma)
was then added to 30%. The protein was concentrated to
100 mg/ml by ultrafiltration in an Amicon stir cell
15 (10,000 MWCO membrane). Concentrated enzyme was
aliquoted and stored at -70°C. Thermolysin was
crystallized by the addition of 9 volumes demineralized
water to 1 volume concentrated (100 mg/ml) protein
solution. The solution was briefly vortexed and
20 allowed to stand overnight at room temperature.
Crystals were washed with 10 volumes of 10 Mm calcium
acetate pH 7.0 and recovered by low speed centrifuga-
tion (10 min at 1500 X G, Beckman GPR centrifuge).
        The rapid addition of water to a concentrated (100
25 mg/ml) solution of thermolysin induces the formation of
crystals which become visible under low-power
magnification within ten minutes. Crystal size is
reproducibly dependent on the final protein concentra-
tion. Three volumes of water to one volume of
30 thermolysin concentrate (100 mg/ml) will produce 0.5mm

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long, X-ray diffraction quality hexagonal rods that
correspond to the crystals described earlier by Colman
et al. (Colman, P.M., Jansonius, J.N. and Matthews,
B.W., J. Mol. Biol. 70: 701-724 (1972)), as confirmed
5   by us by diffraction analysis. Adding ten volumes of
water to one of protein concentrate reduces the length
of the resulting crystals to 0.05mm. These micro-
crystals are preferred in CLEC applications, since they
tend to minimize diffusion problems related to crystal
10 size (see eg., Quiocho, F.A. and Richards, F.M.
Biochemistry 5: 4062-4076 (1967)). Within a given
batch of protein, crystal size was consistently
uniform. (Crystals 0.05 - 0.10 mm in length were used
in this study to facilitate accurate pipetting of
15 crystalline suspensions.) Densitometer scans of SDS-
PAGE showed a six-fold purification of the enzyme on
crystallization, significantly increasing the specific
activity of the CLECs. Crystallization resulted in a
20% decrease in the total activity of the CLEC prot in
20 compared to soluble thermolysin, when assayed by
spectrophotometric cleavage of the dipeptide substrat
furylacryloyl-glycyl-L-leucine-amide (FAGLA), as
described below.

Crosslinking of thermolysin crystals
25   Thermolysin crystals were crosslinked for 3 hours
at room temperature in a solution of 12.5%
glutaraldehyde (Sigma), 5% DMSO and 50 mM Tris pH 6.5.
The crosslinked crystals were washed 3 times in
demineralized water and recovered by low speed

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centrifugation, as described in respect to crystal-
lization of thermolysin. Chemical cross-linking of
enzyme crystals stabilizes the crystal lattice and the
constituent enzyme molecules in the crystal suf-
5 ficiently so as to permit the practical use of CLECs in
environments that are otherwise incompatible with
enzyme function. There was no measurable difference in
enzymatic activity between the crosslinked and un-
crosslinked crystals when assayed (spectrophoto-
10 metrically) by monitoring cleavage of the dipeptide
substrate FAGLA (described below). Moreover, cross-
linking stabilizes CLECs to the point that they can be
lyophilized, with retention of full enzymatic activity
upon reconstitution in aqueous, organic, and mixed
15 aqueous-organic solvents as shown in Figure 1 and Table
8. Although crystallization resulted in a 30% decrease
in the specific activity of the CLEC protein compared
to soluble thermolysin, crosslinking and lyophilization
of the CLECs did not further diminish specific
20 activity.

TABLE 8 - Thermolysin Activity

	Time (min)	Absorbance 345 nm	
		CLEC	Soluble Enzyme
1	0.0	0.314	0.315
2	1.0	0.272	0.271
3	3.0	0.235	0.218
4	5.0	0.204	0.198
5	10.0	0.184	0.185
6	15.0	0.183	0.184

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<u>Enzymatic activity of soluble and CLEC thermolysin</u>

    The catalytic activity of soluble and CLEC
thermolysin was assayed (Feder, J. and Schuck, J.M.,
Biochemistry 9: 2784-2791 (1970)) by hydrolysis of the
5   blocked dipeptide substrate furylacryloyl-glycyl-L-
leucine-amide (FAGLA) (Schweizerhall). Cleavage of th
amide bond was measured spectrophotometrically by a
decrease in absorbance at 345 nm. Initial enzyme
concentration was $10^{-7}M$ by Bradford protein deter-
10 mination and densitometer scanning (Pharmacia LKB
UltroScan XL) of Coomassie stained SDS-PAGE gels. CLEC
enzyme is defined as reconstituted lyophilized cross-
linked thermolysin crystals. Soluble enzyme is defined
as thermolysin concentrated to 100 mg/ml. Enzyme was
15 added to a 5 ml reaction volume containing substrate.
Aliquots of the reaction mix were removed at the
indicated times, and absorbance at 345nm was measured.
CLEC thermolysin was separated from the reaction mix by
brief centrifugation (Beckman, microcentrifuge E)
20 before reading absorbance. Absorbance was fitted to a
pseudo first order rate equation and kcat/Km was
calculated by dividing the fitted value by enzyme
concentration (Multifit 2.0 Curve Fitting for the Apple
Macintosh Computer, Day Computing P.O. Box 327, Milt n,
25 Cambridge CB4 6WL, U.K. (1990)).

<u>pH Dependence and Stability</u>

    The pH optimum and stability of the soluble enzyme
were compared to that of thermolysin CLECs by cleavage
of the dipeptide substrate FAGLA. Results are shown in

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Figure 2 and Table 9. Both soluble and crystalline
enzyme forms demonstrate maximum activity at pH 7.
CLECs and soluble thermolysin also demonstrated similar
activity in the acidic range and the bell shaped pH
5 profile generated by the soluble enzyme was in good
agreement with published data (Feder, J. and Schuck,
J.M., Biochemistry 9: 2784-2791 (1970)). In the
alkaline pH range, however, the crystalline enzyme
maintains maximum activity, to pH 10, while the soluble
10 enzyme has 75% activity at pH 8.5, and only 25%
activity at pH 9. At pH 9.5, the soluble enzyme is
completely inactive.

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TABLE 9 - Thermolysin pH Curve

			% Maximum Activity	
		pH	CLEC	Soluble Enzyme
5	1	5.0	10.250	5.170
	2	5.5	9.750	6.070
	3	6.0	52.500	39.100
	4	6.5	85.000	74.610
	5	7.0	97.500	100.000
10	6	7.5	100.000	98.650
	7	8.0	97.500	82.920
	8	8.5	95.000	71.910
	9	9.0	96.250	24.720
	10	9.5	95.000	0.000
15	11	10.0	90.000	0.000

Stability at elevated temperature

One can achieve higher reaction rates and lower
diffusion times for substrates and products by
operating a given chemical process at higher
20 temperature, where one is usually limited by the
temperature stability of substrates and products. In
enzyme-based catalysis, however, it is often the loss
of enzymatic activity that sets the practical limit on
the temperature that a process can be run. The
25 additional stabilization achieved in CLECs allows for
enzymatic activity at much higher temperatures than is
possible for soluble enzyme.

The enhanced stability at lower temperatures
simplifies the routine long term storage of the CLEC

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catalysts. For example, it was necessary to store
concentrated (>50mg/ml) solutions of soluble
thermolysin at -80°C to retain maximum specific
activity. At room temperature, activity was usually
5   lost within one day. In contrast, rehydrated
thermolysin CLECs could be routinely stored for months
at room temperature with no apparent loss of activity.
Unreconstituted lyophilized CLECs of thermolysin appear
to be viable indefinitely.

10   Thermal stability and resistance to autolysis were
demonstrated in thermolysin CLECs following incubation
at 65°C for five consecutive days (Figure 3 and Table
10). Thermolysin CLECs retained maximum activity after
five days incubation at elevated temperature. In
15   contrast, the soluble thermolysin lost 50% of its
initial activity after only two hours incubation and
demonstrated negligible activity after 24 hours
incubation at 65°C.

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TABLE 10 - Thermolysin Thermal Stability at 65°C

% Maximum Activity

		Time (days)	CLEC	Soluble Enzyme
5	1	0.000	100.000	100.000
	2	0.041		70.000
	3	0.083	96.000	50.000
	4	0.164		32.000
	5	0.246		17.000
10	6	0.410	97.0	10.000
	7	1.000	101.0	2.000
	8	2.000	97.0	
	9	3.000	94.0	
	10	4.000	96.0	
15	11	5.000	92.0	

The activity of soluble and CLEC thermolysin was
measured following incubation at 65°C. Soluble
thermolysin was incubated in 10 mM calcium acetate, 50
mM Tris pH 7.0 in a 65°C water bath. The reaction
20   volume was 500µl. Final protein concentration was 10
mg/ml. Aliquots were removed at times 0, 1, 2, 4, 6,
10, and 18 hours. The samples were assayed by SDS-PAGE
and FAGLA cleavage at room temperature as described
above. For the thermolysin CLECs, a 250 µl crystal
25   suspension in 10mM calcium acetate and 50 mM Tris was
also incubated in a 65°C water bath. Activity was
assayed at times 0, 1, 6, 24, 48, 72, 96, and 120 hours
by FAGLA cleavage.

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Resistance to exogenous proteolysis

Assessment of the resistance of the thermolysin CLEC to the action of an exogenous protease was also carried out. SDS-PAGE (Sodium dodecyl sulfate poly
5 acrylamide gel electrophoresis) analysis suggests that commercial enzymes can contain a substantial percentage of contaminants, some of which might have proteolytic activity against the principal soluble enzyme species. Given the packing of enzyme molecules in a crystal
10 lattice one might assume that the interior enzyme molecules in a CLEC would be protected from proteolysis. To test this possibility, thermolysin CLECs and a soluble enzyme preparation were incubated in the presence of the streptococcal protease,
15 Pronase®, a nonspecific protease capable of digesting most proteins to free amino acids (Calbiochem 1990 Catalog; LaJolla, CA).

Soluble and CLEC thermolysin were incubated in 50mM Tris, pH 7.5, at 40°C in the presence of the
20 protease Pronase® (Calbiochem). The Pronase® to thermolysin ratio was 1/40. To inhibit thermolysin autolysis and prevent the proteolytic destruction of pronase by the thermolysin, EDTA was added to the soluble enzyme reaction to a final concentration of 100
25 mM (EDTA inhibits thermolysin activity but not Pronase®). At the times indicated aliquots were removed from the reaction mix and activity was assayed spectrophotometrically by cleavage of the dipeptide substrates FAGLA. To offset thermolysin inhibition due
30 to the presence of EDTA, the spectrophotometric assay

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of soluble enzyme activity was performed in 0.5 M
calcium acetate buffer pH 7.0 and enzyme concentrati n
was increased two fold. Crosslinked crystalline enzyme
was assayed as described above.

5   As can be seen in Figure 4 and Table 11, the
soluble thermolysin was rapidly degraded and lost all
activity after 90 minutes incubation. In contrast, the
activity of the thermolysin CLEC was unaffected by f ur
days incubation in the presence of protease. This near
10 imperviousness to proteolysis is of particular interest
in diagnostic biosensor applications where a suitable
CLEC might be called upon to act in the presence of an
unknown cocktail of naturally occurring proteolytic
enzymes.

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TABLE 11

Protease Resistance

		Time (days)	% Maximum Activity CLEC	% Maximum Activity Soluble Enzyme	Time (min)
5	1	0.000	100.0	100.0	0.000
	2	0.003		25.0	5.000
	3	0.010		17.5	15.000
	4	0.021		9.5	30.000
	5	0.042	98.0	3.0	60.000
10	6	0.063		1.0	90.000
	7	0.084	101.0	0.0	
	8	1.000	97.0		
	.9	2.000	99.0		
	10	3.000	98.0		
15	11	4.000	96.0		

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of organic solvents. (Table 12). Soluble enzyme
concentrations which could be incubated in organic
solvent were limited to a maximum of 10 mg/ml.
Concentrations greater than this value resulted in the
5 instantaneous precipitation of thermolysin upon
addition of organic solvent. In contrast, thermolysin
CLEC concentrations were limited only by the volume
occupied by the crystals. Soluble thermolysin retained
the greatest activity (75%) following incubation in
10 acetone, and the least (36%) in tetrahydrofuran.
Following a one hour incubation in the presence of
acetonitrile or dioxane the soluble enzyme lost
approximately 50% of its initial activity. The CLEC
thermolysin retained greater than 95% maximum activity
15 following incubation with all organics assayed.
TABLE 12

	% Maximum Activity	
	Soluble Enzyme	CLEC
20 Acetonitrile	42	102
Dioxane	66	97
Acetone	75	99
THF*	36	96

* Tetrahydro Furan

25 <u>Stability in organic solvents</u>
    Thermolysin CLECs or soluble thermolysin
preparations were incubated in 50% (v/v) solutions of

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the indicated rganic solvents. A 100 μl slurry of
thermolysin CLECs (10 mg/ml) in 10mM Tris pH 7 was
placed in a 1/2 dram glass vial. An equal volume of
the indicated organic solvent was added and the mixture
5   was briefly vortexed. Twenty μl of soluble thermolysin
(100 mg/ml) was diluted in 80 μl of 0.015M Tris buffer
pH 7.0 in a 1/2 dram glass vial. A 100 μl volume of
organic solvent was then added to the protein solution
and briefly vortexed. CLEC and soluble enzyme were
10 incubated in the presence of organic solvent for one
hour at 40°C. Following incubation, enzyme activity
was assayed by cleavage of the dipeptide substrate
FAGLA as described.

Low water concentration is thought to disfavor
15 unfolding to intermediate states on the path to enzyme
denaturation. In CLECs, this restriction of
conformational mobility is provided by the inter-.
molecular contacts and cross-links between the
constituent enzyme molecules making up the crystal
20 lattice, rather than by the near-absence of water in
the medium. As a result, intermediate water-organic
solvent concentrations are readily tolerated by enzymes
when formulated as CLECs, something previously
unobserved with enzymes (see Table 12). This discovery
25 opens up whole new areas of synthetic chemistry to
exploitation using enzyme catalysis.

Even in near-anhydrous organic solvents, however,
the routine use of enzymes has been hampered by their
tendency to form ill-defined suspensions that are
30 subject to clumping and other aggregation problems.

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This pr perty makes these preparations inherently
unattractive for large scale industrial processes. In
contrast, CLECs and the constituent enzymes within the
crystal lattice, remain mono-disperse in all these
5 solvents.

Comparison with other immobilization methods

A number of useful reviews of enzyme
immobilization methods have appeared in the literature
(Maugh, T.H., Science, 223: 474-476 (1984)); Tramper,
10 J., Trends in Biotechnology 3: 45-50 (1985)) . · In
these, the enzyme always represents a small fraction of
the total volume of the immobilized particle, the bulk
of it being inert carrier material. The carrier
increases the mean free path between the solvent
15 exterior of the immobilized enzyme particle and the
enzyme active sites, exacerbating diffusion problems
(Quiocho, F.A. and Richards, F.M., Biochemistry 5:
4062-4076 (1967)).

In a CLEC, the cross-linked crystal matrix
20 provides its own support, eliminating the need for a
carrier. As a result, the concentration of enzyme in a
CLEC is close to the theoretical packing limit that can
be achieved for molecules of a given size, greatly
exceeding densities achievable even in concentrated
25 solutions. The entire CLEC consists of active enzym ,
and thus, the diffusion-related reduction of enzyme
reaction rates usually observed with conventionally
immobilized enzymes relative to enzymes in solution are
minimized (See Figure 1), since the mean free path f r

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substrate and pr duct between active enzyme and free
solvent will b.a greatly shortened for CLECs (compared
to a conventional immobilized enzyme carrier
particles). Importantly, the constituent enzyme in
5   CLECs is intrinsically mono-disperse, and can be
recovered by simple manipulations of the CLEC
particles, such as filtration, centrifugation or
decantation of solvent.

     EXAMPLE 3
10   Crystallization, cross-linking and lyophilization of
elastase and assessment of characteristics of the
resulting product.
Crystallization of elastase
     Lyophilized porcine pancreatic elastase (Serva)
15   was dissolved in 0.1 M sodium acetate pH 5.0 to a
concentration of 5 mg/ml (w/v) at room temperature.
Rod shaped elastase crystals were visible within one
minute of the complete solvation of the protein. The
crystallization solution was transferred to 4°C and
20   crystallization was completed overnight. Crystals were
recovered by centrifugation as previously described.

Cross-linking of elastase crystals
     A 200 µl volume of elastase crystals was added to
a 1.3 ml solution of 5.77% glutaraldehyde and 1.5 M
25   sodium acetate pH 5.0. The crystals were crosslinked
for one hour with mild agitation (stir plate).
Following cross-linking the crystals were washed with
three 15 ml volumes of 0.2 M Tris pH 8.0. The elastase

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CLEC was lyophilized as described in Example 2.

Enzymatic activity of soluble and CLEC elastase

     The catalytic activity of soluble and CLEC
elastase was assayed spectrophotometrically by
5   measuring hydrolysis of the substrate succinyl - (Ala)₃
p-nitroanilide (Bachem) [Bieth, et al. Biochem. Med
11:350-357 (1974)] (Table 13 Figure 5). Cleavage was
monitored by increasing absorbance at 410 nm. Initial
substrate concentration was 2×10^{-4}. Enzyme
10 concentration was $2.8 \times 10^{-7} M$. CLEC or soluble enzyme
was added to a 5 ml reaction volume containing
substrate in 0.2 M Tris pH 8.0. As described
previously, CLEC enzyme was removed from the reaction
mix prior to measuring absorbance.

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TABLE 13 - Elastase Activity

Absorbance 400 nm

		Time (min)	CLEC	Soluble Enzyme
5	1	0.0	0.000	0.000
	2	0.5	0.103	0.205
	3	1.0	0.195	0.390
	4	2.0	0.366	0.672
	5	3.0	0.523	0.923
10	6	4.0	0.657	1.098
	7	5.0	0.780	1.227
	8	6.0	0.888	1.326
	9	7.0	0.974	1.393
	10	10.0	1.170	1.512
15	11	15.0	1.365	1.586

Resistance to exogenous proteolysis

Assessment of the resistance of the elastase CLEC
to the action of protease was also performed under
identical conditions as described for thermolysin
20 (Example 2). Activity of the soluble and CLEC enzyme,
following incubation with protease, was assayed by
hydrolysis of the nitroanilide substrate as described
above (Table 14 and Figure 6).

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TABLE 14 - Elastase Resistance to Proteolysis

% Maximum Activity

		Time	CLEC	Soluble Enzyme
	1	0.0	100.0	100.0
5	2	10.0		53.0
	3	20.0		32.0
	4	30.0	101.0	18.0
	5	45.0		11.0
	6	60.0	102.0	8.0
10	7	120.0	101.0	3.0
	8	180.0	103.0	2.0

Example 4
Crystallization, cross-linking and lyophilization of
esterase and assessment of characteristics of the
15 resulting product.
Crystallization of esterase

As disclosed here, 30 mg/ml ammonium sulfate
suspension of pig liver esterase (Fluka) was dissolved
in 0.25 M calcium acetate pH 5.6 at room temperature.
20 Esterase crystals were visible within several minutes
following addition of the calcium acetate solution.
The crystallization solution was allowed to stand at
room temperature and crystallization was completed
overnight. Crystals were recovered by centrifugati n
25 as previously described in Example 2.

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Cross-linking of esterase crystals

      As disclosed here, a 300 μl volume of esterase
crystals were added to a 5 ml solution of 12.5%
glutaraldehyde and 0.5 M sodium acetate pH 5.5. The
5   crystals were crosslinked for one hour with mild
agitation (stir plate). Following cross-linking the
crystals were washed with three 15 ml volumes of 0.5 M
calcium acetate pH 6.3. The esterase CLEC was
lyophilized as previously described in Example 2.

10   Enzymatic activity of soluble and CLEC esterase

      The catalytic activity of soluble and CLEC
esterase was assayed spectrophotometrically by
monitoring hydrolysis of the substrate p-nitrophenyl
acetate (Fluka) (Table 15 and Figure 7). Cleavage was
15   monitored by increasing absorbance at 400 nm. Initial
substrate concentration was 0.001%. Enzyme
concentration was 1 X 10^{-4}M. CLEC or soluble enzyme was
added to a 5 ml reaction volume containing substrate in
0.25 M calcium acetate pH 6.3. As described previously
20   in example 2, CLEC enzyme was removed from the reaction
mix by centrifugation prior to measuring absorbance.

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TABLE 15 - Esterase Activity

Absorbance 400 nm

		Time (min)	CLEC	Soluble Enzyme
5	1	0.0	0.000	0.000
	2	0.5	0.770	0.252
	3	1.0	0.128	0.297
	4	2.0	0.208	0.337
	5	3.0	0.260	0.346
10	6	5.0	0.324	0.353
	7	7.0	0.353	0.359
	8	10.0	0.369	0.368

Resistance to exogenous proteolysis

Assessment of the resistance of the esterase CLEC
15 to the action of protease was also performed under
identical conditions as described for thermolysin
(Example 2). Activity of the soluble and CLEC enzyme,
following incubation with protease, was assayed by
hydrolysis of the substrate p-nitrophenyl acetate as
20 described above (Table 16 and Figure 8).

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TABLE 16 - Esterase Resistance to Proteolysis

			% Maximum Activity	
		Time (min)	CLEC	Soluble Enzymes
5	1	0.0	100.0	100.0
	2	10.0		68.0
	3	20.0		47.0
	4	30.0	99.0	25.0
	5	45.0		20.0
10	6	60.0	97.0	16.0
	7	120.0	94.0	10.0
	8	180.0	91.0	6.0

Example 5
Crystallization, cross-linking and lyophilization of
15 **lipase and assessment of characteristics of the**
resulting product.
Crystallization of lipase

     As disclosed here, the enzyme lipase (Geotrichum
candidum) was crystallized by vapor diffusion from an
20 aqueous solution of 20 mg/ml protein in 50 mM Tris pH 7
containing 8% ammonium sulfate. Bipyrimidal crystals
were visible after 20 to 30 days incubation at room
temperature. Crystals were recovered by
centrifugation, as previously described in Example 2.

25 **Cross-linking of lipase crystals**

     As disclosed here, lipase crystals were added to a
solution of 12.5% glutaraldehyde and 50 mM Tris pH 5.6.

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The crystals were crosslinked for one hour. Foll wing
cross-linking the crystals were washed with three 15 ml
volumes of 50 mM Tris pH 7.0. The lipase CLEC was
lyophilized, as previously described in Example 2.

5 <u>Enzymatic activity of soluble and CLEC lipase</u>

       The catalytic activity of soluble and CLEC lipase
was assayed spectrophotometrically by monitoring
hydrolysis of the substrate p-nitrophenyl acetate
(Table 17 and Figure 9). Cleavage was monitored by
10 increasing absorbance at 400 nm. Initial substrate
concentration was 0.005%. Enzyme concentration was 1.5
X 10^{-3}M. CLEC or soluble enzyme was added to a 5 ml
reaction volume containing substrate in 0.2 M Tris pH
7.0 at room temperature. As described previously in
15 Example 2, CLEC enzyme was removed from the reacti n
mix by centrifugation prior to measuring absorbance.

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TABLE 17 - Lipase Activity

Absorbance 400 nm

		Time (min)	CLEC	Soluble Enzyme
5	1	0.0	0.000	0.000
	2	1.0	0.013	0.021
	3	5.0	0.094	0.116
	4	10.0	0.164	0.186
	5	15.0	0.248	0.258
10	6	30.0	0.346	0.357
	7	45.0	0.407	0.420
	8	60.0	0.461	0.459
	9	90.0	0.497	0.502

Example 6

15 Crystallization, cross-linking and lyophilization of
lysozyme and assessment of characteristics of the
resulting product
Crystallization of lysozyme

Following the method of Blake, C.C.F. et al.,
20 Nature 196:1173 (1962) 200 mg of lyophilized hen egg
white lysozyme (Boehringer Mannheim) was dissolved in
2.5 ml of 0.04 M sodium acetate buffer pH 4.7 at room
temperature. Following solvation of the protein, 2.5
ml of 10% sodium chloride were added to the lysozyme
25 solution dropwise with stirring. The crystallization
solution was allowed to stand overnight at room
temperature, and crystallization was completed in

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forty-eight hours. Crystals were recovered by
centrifugation, as previously described in Example 2.

Cross-linking of lysozyme crystals

        As described here, a 500 μl volume of lysozyme
5   crystals was added to 10 ml of 24% glutaraldehyde and
50 mM Tris pH 5.6 containing 20% sodium chloride. The
crystals were crosslinked for 20 minutes with mild
agitation (stir plate). Following cross-linking the
crystals were washed with three 50 ml volumes of 20 mM
10 calcium acetate and 50 mM potassium chloride pH 5.3.
The lysozyme CLEC was lyophilized, as previously
described in Example 2.

Enzymatic activity of soluble and CLEC lysozyme

        The catalytic activity of soluble and CLEC
15 lysozyme was assayed by measuring the rate of
hydrolysis of the substrate 4-methylumbelliferyl N-
acetyl-chitrioside (Fluka) (Yang, Y. and Hamaguchi, K.
J. Biochem. 8:1003-1014 (1980) (Table 18 and Figure
10). The release of 4-methylumbelliferone was followed
20 fluorimetrically (Perkin Elmer Model LS-50). Initial
substrate concentration was 1.42 X 10^{-3}. Enzyme
concentration was 3 X 10^{-7}. CLEC or soluble enzyme was
added to a 2 ml reaction volume containing substrat in
20 mM calcium acetate and 50 mM potassium chloride pH
25 5.3 at 42°C. The amount of 4-methylumelliferone was
determined fluorimetrically by measuring fluorescence
intensities at 450 nm with excitation at 360 nm. Slit
width for both excitation and emission was 10 mm. As

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described previously in Example 2, CLEC enzyme was removed from the reaction mix by centrifugation pri r to measuring fluorescence.

TABLE 18 - Lysozyme Activity

		Fluorescence	
	Time (min)	CLEC	Soluble Enzyme
1	0.000	0.000	0.000
2	10.000	4.400	18.900
3	30.000	10.500	29.400
4	60.000	27.500	44.800
5	90.000	33.800	51.700
6	120.000	45.900	59.800

Example 7

Crystallization, cross-linking and lyophilization of asparaginase and assessment of characteristics of the resulting product.

Crystallization of asparaginase

As a modification of the procedure described by Grabner et al. [U.S. Patent 3,664,926 (1972)] 25 mg of lyophilized asparaginase (Worthington) were dissolved in 500 μl of 50 mM sodium phosphate buffer pH 7.2. The solution was cooled to 4°C and the pH adjusted to 5.0 with 1 M acetic acid. Cold (-20°C) ethanol was then added dropwise to the asparaginase solution to a final concentration of 33%. The solution was incubated at 4°C. Crystallization was completed in forty-eight hours. Crystals were recovered by centrifugation as previously described.
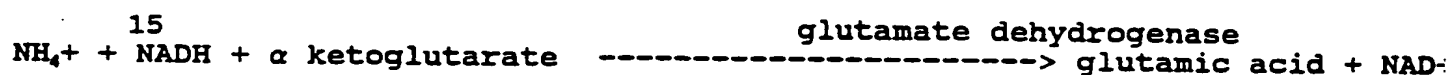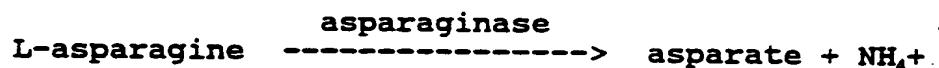
Cross-linking of asparaginase crystals

As disclosed here, asparaginase crystals were

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crosslinked in a solution of 7.5% glutaraldehyde in 50
mM sodium phosphate buffer pH 5.6.   Following
crosslinking the crystals were washed with five 15 ml
volumes of 50 mM tris pH 7.0.   The asparaginase CLECs
5   were lyophilized, as previously described in Example 2.

Enzymatic activity of soluble and CLEC asparaginase

        The catalytic activity of soluble and CLEC
asparaginase was assayed spectrophotometrically by
measuring evolution of ammonium ion in the coupled
10   enzymatic reaction described below (all reagents were
purchased from Boehringer Mannheim) (Table 19 and
Figure 11).

$$\text{L-asparagine} \xrightarrow{\text{asparaginase}} \text{asparate} + NH_4^+$$

15

$$NH_4^+ + NADH + \alpha \text{ ketoglutarate} \xrightarrow{\text{glutamate dehydrogenase}} \text{glutamic acid} + NAD$$

        Oxidation of NADH was measured by decreasing absorbance
at 340 nm.   Initial NADH concentration was 1.4 mg/ml.
Asparagine concentration was 10^{-3}M.   Alpha ketoglutarate
20   concentration was 10^{-4}M.   Glutamate dehydrogenase
concentration was 10^{-7}M. ·Asparaginase concentration was
2.3 x 10^{-8}M.   As described previously in Example 2, CLEC
enzyme was removed from the reaction mix by
centrifugation prior to measuring absorbance.

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TABLE 19 - Asparaginase Activity

Absorbance 340 nm

		Time (min)	CLEC	Soluble Enzyme
5	1	0.0	1.000	1.000
	2	1.0	0.867	0.825
	3	3.0	0.739	0.684
	4	5.0	0.603	0.538
	5	10.0	0.502	0.406
10	6	15.0	0.449	0.338
	7	30.0	0.328	0.199
	8	45.0	0.211	0.187

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CLAIMS

1) An enzyme-catalyzed method of making a selected product, comprising:

    a)   combining at least one appropriately-selected
5   substrate and at least one enzyme which acts
         upon the substrate and is in the form of a
         cross-linked immobilized enzyme crystal; and

    b)   maintaining the combination produced in step
         (a) under conditions appropriate for the
10   enzyme to act upon the substrate, thereby
         producing the selected product.

2) A method of Claim 1 wherein the selected product
is selected from the group consisting of:
peptides, lipids, chiral organic molecules and
15 carbohydrates.

3) An enzyme-catalyzed method of Claim 1 wherein the
cross-linked immobilized enzyme crystal is
approximately 10^{-1} mm. in size.

4) An enzyme-catalyzed method of Claim 1 wherein the
20 conditions in step (b) are deleterious to the
efficient function of the enzyme which is not in
the form of a cross-linked immobilized enzyme
crystal.

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5) A method f Claim 4 wherein th c nditions in step
(b) are selected from the group consisting of high
temperature, low pH, high pH, organic solvents
mixed aqueous/organic, and near anhydrous

5   conditions.

6) An enzyme-catalyzed method of making a selected
product, comprising:
   a)   combining two appropriately-selected
        substrates and at least one enzyme which acts
10   upon at least one of the appropriately-
        selected substrates and is in the form of a
        cross-linked immobilized enzyme crystal; and
   b)   maintaining the combination produced in step.
        (a) under conditions appropriate for the
15   enzyme to act upon at least one of the
        appropriately-selected substrates, thereby
        producing the selected product.

7) A method of Claim 6 wherein the selected product
   is selected from the group consisting of:
20   peptides, lipids, chiral organic molecules and
   carbohydrates.

8) An enzyme-catalyzed method of Claim 6 wherein the
   cross-linked immobilized enzyme crystal is
   approximately 10^{-1} mm. in size.

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9)   An enzyme-catalyzed method of Claim 6 wherein the
      conditions in step (b) are deleterious to the
      efficient function of the enzyme which is not in
      the form of a cross-linked immobilized enzyme
5   crystal.

10)   A method of Claim 6 wherein the conditions in step
      (b) are selected from the group consisting of high
      temperature, low pH, high pH, organic solvents
      mixed aqueous/organic and near anhydrous
10   conditions.

11)   An enzyme-catalyzed method of making a selected
      product in an organic solvent, comprising:
      a)   combining at least one appropriately-selected
            substrate; at least one enzyme which acts
15   upon the substrate and is in the form of a
            cross-linked immobilized enzyme crystal; and
            the organic solvent; and
      b)   maintaining the combination produced in step
            (a) under conditions appropriate for the
20   enzyme to act upon the substrate, thereby
            producing the selected product.

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12) An enzyme-catalyzed meth d f producing a selected
product, comprising combining an
appropriately-selected substrate and a
cross-linked immobilized enzyme crystal which acts

5   upon the appropriately-selected substrate, under
conditions appropriate for the cross-linked
immobilized enzyme crystal to act upon the
appropriately-selected substrate, thereby altering
the substrate and producing the selected product.

10 13) A method of producing aspartame comprising the
steps of: a) combining two peptides and
cross-linked immobilized thermolysin crystals, a
first peptide having the formula Z-L-Asp, a second
peptide having the formula DL-Phe-OMe, under

15   conditions appropriate for condensation of the two
peptides through action of the cross-linked
immobilized enzyme crystal, thereby producing a
partially protected dipeptide of the formula
Z-L-Asp-L-Phe-OMe, wherein Z represents a

20   benzyloxycarbonyl group, and b) removing the
benzyloxycarbonyl group, thereby producing
aspartame.

14) A method of immobilizing an enzyme and retaining
its activity, comprising providing the enzyme as a

25   crystal and cross-linking the crystal, thereby
producing a cross-linked immobilized enzyme
crystal of approximately 10^{-1} mm in size.

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15) A cross-linked immobilized enzyme crystal
     approximately 10^{-1} mm in size produced by the
     method of Claim 14.

16) A cross-linked immobilized enzyme crystal of Claim
5   15, selected from the group consisting of:
     thermolysin, lipases, elastases, esterases,
     lysozymes and asparaginase.

17) The method of Claim 14, additionally comprising
     lyophilizing cross-linked immobilized crystals
10   produced.

18) A lyophilized cross-linked immobilized enzyme
     crystal produced by the method of Claim 17.

19) A lyophilized cross-linked immobilized enzyme
     crystal of Claim 18 selected from the group
15   consisting of: thermolysin, lipases, esterases,
     elastases, lyosozymes and asparaginase.

20) A method of immobilizing thermolysin and retaining
     its activity , comprising providing thermolysin
     crystals and cross-linking the thermolysin
20   crystals, thereby producing cross-linked
     immobilized thermolysin crystals approximately 10^{-1}
     mm in size.

21) Cross-linked immobilized crystals of thermolysin
     produced by the method of Claim 20.

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TABLE 19 - Asparaginase Activity

Absorbance 340 nm

		Time (min)	CLEC	Soluble Enzyme
5	1	0.0	1.000	1.000
	2	1.0	0.867	0.825
	3	3.0	0.739	0.684
	4	5.0	0.603	0.538
	5	10.0	0.502	0.406
10	6	15.0	0.449	0.338
	7	30.0	0.328	0.199
	8	45.0	0.211	0.187

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<center>**CLAIMS**</center>

1)   An enzyme-catalyzed method of making a selected product, comprising:

    a)   combining at least one appropriately-selected
5   substrate and at least one enzyme which acts upon the substrate and is in the form of a cross-linked immobilized enzyme crystal; and

    b)   maintaining the combination produced in step (a) under conditions appropriate for the
10   enzyme to act upon the substrate, thereby producing the selected product.

2)   A method of Claim 1 wherein the selected product is selected from the group consisting of: peptides, lipids, chiral organic molecules and
15   carbohydrates.

3)   An enzyme-catalyzed method of Claim 1 wherein the cross-linked immobilized enzyme crystal is approximately 10^{-1} mm. in size.

4)   An enzyme-catalyzed method of Claim 1 wherein the
20   conditions in step (b) are deleterious to the efficient function of the enzyme which is not in the form of a cross-linked immobilized enzyme crystal.

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5) A method f Claim 4 wherein the conditions in step
(b) are selected from the group consisting of high
temperature, low pH, high pH, organic solvents
mixed aqueous/organic, and near anhydrous
conditions.

6) An enzyme-catalyzed method of making a selected
product, comprising:
a) combining two appropriately-selected
substrates and at least one enzyme which acts
upon at least one of the appropriately-
selected substrates and is in the form of a
cross-linked immobilized enzyme crystal; and
b) maintaining the combination produced in step
(a) under conditions appropriate for the
enzyme to act upon at least one of the
appropriately-selected substrates, thereby
producing the selected product.

7) A method of Claim 6 wherein the selected product
is selected from the group consisting of:
peptides, lipids, chiral organic molecules and
carbohydrates.

8) An enzyme-catalyzed method of Claim 6 wherein the
cross-linked immobilized enzyme crystal is
approximately 10^{-1} mm. in size.

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9) An enzyme-catalyzed method of Claim 6 wherein the conditions in step (b) are deleterious to the efficient function of the enzyme which is not in the form of a cross-linked immobilized enzyme

5   crystal.

10) A method of Claim 6 wherein the conditions in step (b) are selected from the group consisting of high temperature, low pH, high pH, organic solvents mixed aqueous/organic and near anhydrous

10   conditions.

11) An enzyme-catalyzed method of making a selected product in an organic solvent, comprising:
   a) combining at least one appropriately-selected substrate; at least one enzyme which acts

15   upon the substrate and is in the form of a cross-linked immobilized enzyme crystal; and the organic solvent; and
   b) maintaining the combination produced in step (a) under conditions appropriate for the

20   enzyme to act upon the substrate, thereby producing the selected product.

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12) An enzyme-catalyzed method of producing a selected
product, comprising combining an
appropriately-selected substrate and a
cross-linked immobilized enzyme crystal which acts
5   upon the appropriately-selected substrate, under
conditions appropriate for the cross-linked
immobilized enzyme crystal to act upon the
appropriately-selected substrate, thereby altering
the substrate and producing the selected product.

10 13) A method of producing aspartame comprising the
steps of: a) combining two peptides and
cross-linked immobilized thermolysin crystals, a
first peptide having the formula Z-L-Asp, a second
peptide having the formula DL-Phe-OMe, under
15   conditions appropriate for condensation of the two
peptides through action of the cross-linked
immobilized enzyme crystal, thereby producing a
partially protected dipeptide of the formula
Z-L-Asp-L-Phe-OMe, wherein Z represents a
20   benzyloxycarbonyl group, and b) removing the
benzyloxycarbonyl group, thereby producing
aspartame.

14) A method of immobilizing an enzyme and retaining
its activity, comprising providing the enzyme as a
25   crystal and cross-linking the crystal, thereby
producing a cross-linked immobilized enzyme
crystal of approximately 10^{-1} mm in size.

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15) A cr ss-linked immobilized enzyme crystal approximately 10^{-1} mm in size produced by the method of Claim 14.

16) A cross-linked immobilized enzyme crystal of Claim 15, selected from the group consisting of: thermolysin, lipases, elastases, esterases, lysozymes and asparaginase.

17) The method of Claim 14, additionally comprising lyophilizing cross-linked immobilized crystals produced.

18) A lyophilized cross-linked immobilized enzyme crystal produced by the method of Claim 17.

19) A lyophilized cross-linked immobilized enzyme crystal of Claim 18 selected from the group consisting of: thermolysin, lipases, esterases, elastases, lyosozymes and asparaginase.

20) A method of immobilizing thermolysin and retaining its activity , comprising providing thermol,_in crystals and cross-linking the thermolysin crystals, thereby producing cross-linked immobilized thermolysin crystals approximately 10^{-1} mm in size.

21) Cross-linked immobilized crystals of thermolysin produced by the method of Claim 20.

22) The method of Claim 20, additionally comprising lyophilizing cross-linked immobilized enzyme crystals produced.

23) A lyophilized cross-linked immobilized thermolysin crystal produced by the method of Claim 20.

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24) A device comprising an enzyme in the form of a cross-linked immobilized enzyme crystal and a retaining means for the cross-linked immobilized enzyme crystal, the retaining means consisting of a material which allows contact between the cross-linked immobilized enzyme crystal and a substrate upon which the enzyme acts, the substrate present in a fluid.

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28) A biosensor f r d tecting an analyte of interest
    in a fluid, comprising:
    a)   a cross-linked immobilized luciferase
          crystal, in which the luciferase acts on the
    5   analyte of interest or on a product of a
          reaction in which the analyte of interest
          participates; and
    b)   a retaining means for the cross-linked
          immobilized luciferase crystal, the retaining
    10   means consisting of a material which allows
          contact between the cross-linked immobilized
          luciferase crystal and a fluid containing:
          (1) the analyte upon which the luciferase
          acts or (2) the product of a reaction in
    15   which the analyte participates.

29) An extracorporeal device for altering a component
    of a fluid comprising:
    a)   a cross-linked immobilized enzyme crystal, in
          which the enzyme present acts on the
    20   component or on a reactant in a reaction in
          which the component participates; and
    b)   a retaining means for the cross-linked
          immobilized enzyme crystal, the retaining
          means consisting of a material which allows
    25   contact between the cross-linked immobilized
          enzyme crystal and: (1) the component upon
          which the enzyme acts, the component present
          in the fluid or (2) the product of a reaction
          in which the component participates, the
    30   reactant present in a fluid.

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25) A biosens r f r detecting an analyte of interest
in a fluid, comprising:

a) a cross-linked immobilized enzyme crystal, in
which the enzyme present acts on the analyte
of interest or on a reactant in a reaction in
which the analyte of interest participates;
and

b) a retaining means for the cross-linked
immobilized enzyme crystal, the retaining
means consisting of a material which allows
contact between the cross-linked immobilized
enzyme crystal and a fluid containing: (1)
the analyte upon which the enzyme acts, the
analyte present in a fluid or (2) a reactant
in a reaction in which the analyte
participates, the reactant present in the
fluid.

26) A biosensor of claim 25 further comprising a
detecting means.

27) A biosensor of Claim 25 for detecting an analyte
of interest in a fluid, the analyte of interest
selected from the group consisting of: glucose,
creatinine, urea, lactate, glucose-6- phosphate,
sucrose, ATP, ethanol, acetic acid, formic acid,
cholesterol, uric acid, methotrexate, carbon
dioxide, amino acids, phosphates, penicillin,
nitrates, nitrites, sulphates and succinate.

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30'   An extracorporeal d vice of Claim 29 for altering
      a component of a fluid wherein the component is
      selected from the group consisting of: asparagine,
      heparin, bilirubin, methotrexate, amino acids,
5   urea and ammonia.

31) A device useful for producing a selected product,
     comprising an enzyme in the form of a
     cross-linked immobilized enzyme crystal and a
     retaining means for the cross-linked immobilized
10   enzyme crystal.

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